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Semisynthetic Lantibiotics - Simplicity, Stability, Specificity

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Declaration

I, Serena Mitchell, confirm the work presented in this thesis is my own. Where information has been derived from other sources, I confirm this has been indicated in the thesis.

Signed:

Date:

Abstract

Lantibiotics are a powerful class of antimicrobials but their success in clinical settings has been limited. Stability and solubility issues in combination with the significant synthetic challenge posed by the characteristic thioether ring structures have hampered our ability to exploit this class of natural products.

A well-studied lantibiotic, nisin, is of interest due to its broad-spectrum activity and novel mode of action. It has been shown that rings A and B of nisin bind tightly to lipid II, a precursor for bacterial cell wall biosynthesis. The C terminus can then insert itself into the bacterial membrane to form stable pores causing cell lysis.

This thesis investigates the synthesis of analogues of nisin with simplified structures that maintain good biological activity whilst improving stability, solubility and also giving easier synthetic access to compounds with nisin's unique mode of action. Orthogonally protected lanthionine was synthesised and used in SPPS to produce novel analogues of rings A and B with improved stability. Rings A and B of wild type nisin were also obtained through digestion of a commercially available preparation. To maintain the pore forming abilities of nisin but simplify the synthetic route we have replaced the lanthionine containing C terminus with simpler, linear pore forming peptides and a peptoid and connected these to rings A and B using click chemistry. These hybrid peptides were assessed for their biological activity using a broth microdilution assay.

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Abbreviations

$[\alpha]_D$	Specific rotation
Å	Angstrom
δ_H	Proton (1H) NMR Chemical Shift
δ_C	Carbon (13C) NMR Chemical Shift
°C	Degrees Celsius
µM	Micromolar
µW	Microwave
Aib	α-Aminoisobutyric Acid
Alloc	Allyloxycarbonyl
AMP	AMP
Ar	Aryl
Boc	*tert*-Butoxycarbonyl
BOP	(Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate
Bn	Benzyl
Br	Broad
*n*Bu	Butyl
*t*Bu	*Tertiary* butyl
c	Concentration
Cbz	Carboxybenzyl
d	Doublet
DCC	*N,N'*-Dicyclohexylcarbodiimide
DEAD	Diethyl Azodicarboxylate
DIC	*N,N'*-Diisopropylcarbodiimide
Dha	2,3-Dehydroalanine
Dhb	2,3-Dehydrobutyrine
DIPEA	N,N-Diisopropylethylamine
DMAP	Dimethylamino pyridine
DMF	Dimethylformamide
DTT	1,4-Dithiothreitol
EDCI	*N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide Hydrochloride

ES^x	Electrospray Ionisation
Eq.	Equivalents
Et	Ethyl
Fmoc	9-Fluorenylmethoxycarbonyl
g	Gram
h	Hour
HBTU	*O*-(Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate
HOAt	1-Hydroxy-7-azabenzotriazole
HOBt	1-Hydroxybenzotriazole
HPLC	High Performance Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
Hz	Hertz
J	Coupling Constant
L	Litre
Lan	Lanthionine
LC-MS	Liquid Chromatography-Mass Spectrometry
LRMS	Low Resolution Mass Spectrometry
LUV	Large Unilamellar Vesicle
M	Metre
M	Molar (mol dm^{-3})
m	Multiplet
M^+	Molecular Ion
$M+H^+$	Protonated Molecular Ion
$M-H^-$	Deprotonated Molecular Ion
MBA	(*S*)-(-)-α-Methylbenzylamine
Me	Methyl
MeLan	Methyllanthionine
min	Minute
mL	Millilitre
mM	Millimolar
mol	Mole
mmol	Millimole
MS	Mass Spectrometry
m/z	Mass to Charge Number Ratio

NaAsc	Sodium Ascorbate
nm	Nanometre
NMM	4-Methylmorpholine
NMP	Methylpyrrolidone
NMR	Nuclear Magnetic Resonance Spectroscopy
Nspe	N-(*S*)-(1-Phenylethyl)glycine
Nz	Nitrobenzyloxycarbonyl
o	Ortho
OMe	Methoxy
p	*para*
p-NB	*para*-Nitrobenzyl
p-TSA	*para*-Toluenesulfonic Acid Monohydrate
PEG	Poly(ethylene glycol)
Ph	Phenyl
ppm	Parts Per Millions
PyAOP	(7-Azabenzotriazol-1-yloxy)-tris(pyrrolidino)-phosphonium Hexafluorophosphate
PyBOP	(Benzotriazol-1-yloxy)tripyrrolidinophosphonium Hexafluorophosphate
q	Quartet
s	Singlet
SPPS	Solid Phase Peptide Synthesis
t	Triplet
TBAF	Tetra-n-butylammonium Fluoride
TBTA	Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine
Tce	2,2,2-Trichloroethanol
Teoc	β-(Trimethylsilyl)ethoxycarbonyl
Teoc-OSu	1-[2-(Trimethylsilyl)ethoxycarbonyloxy]pyrrolidone-2,5-dione
tert	Tertiary
TFA	Trifluoroacetic Acid
TFFH	N,N,N',N'-Tetramethyl Formamidinium Hexafluorophosphate
TFMSA	Triflic Acid
THF	Tetrahydrofuran
TIPS	Triisopropylsilane
TLC	Thin Layer Chromatography

TMSCl	Trimethlsilyl Chloride
Trityl	Triphenylmethyl
UV	Ultraviolet
WT	Wild Type

1 Introduction

The rise and spread of resistance to antibiotics and other antimicrobials is well established as one of the greatest threats to modern health. In 2014 a UK government commissioned report estimated that every year 700,000 deaths can be attributed to resistant infections from bacteria and other microbes such as HIV, tuberculosis and malaria.[1] The report went on to estimate that this could rise to 10 million deaths annually by 2050. Whilst the accuracy of these estimates has been contested[2] the severity of the problem and the urgent need for solutions cannot be dismissed. The last few years have seen a rise in bacterial infections that are resistant to even last line of defence antibiotics such as vancomycin and carbapenems. Data collected from US hospitals from 1995 to 2002 showed an increasing proportion of hospital acquired bloodstream infections were due to antibiotic-resistant bacteria (*Figure 1-1*).[3] Given this, we now face the bleak prospect of the occurrence of bacterial infections that are resistant to every available therapeutic and reports of resistance to every available therapeutic.

It is important to note that this is a global problem that requires a globally coordinated response. There is limited data on the extent of antimicrobial resistance in developing countries but it is largely understood that they have the highest rates of mortality due to antimicrobial resistance. This is attributed to several factors including: high rates of infectious disease; weak healthcare systems; limited access to new or expensive drugs; and lack of diagnostic resources for recognising and containing resistant infections. Even when comparing developed countries, high income inequality has been linked to higher rates of antimicrobial resistance.[4]

Current outbreaks of resistance need to be mitigated through global health policy and funding that facilitates: stringent clinical hygiene standards; a cut down of the misuse of antibiotics in humans and livestock; standardization of resistance diagnostics and data collection. Another of the tools essential to tackling resistance is the discovery and development of new antimicrobials with, importantly, novel mechanisms of action. Several drug discovery initiatives have been launched for the development of antibiotics, on both a national and international level,[5] but we are sadly still seeing a decrease in the number of novel drugs reaching the market each year. The pressure for new antibiotics is driving a

resurgence of interest in natural products and the re-evaluation of existing classes of compounds.[6,7]

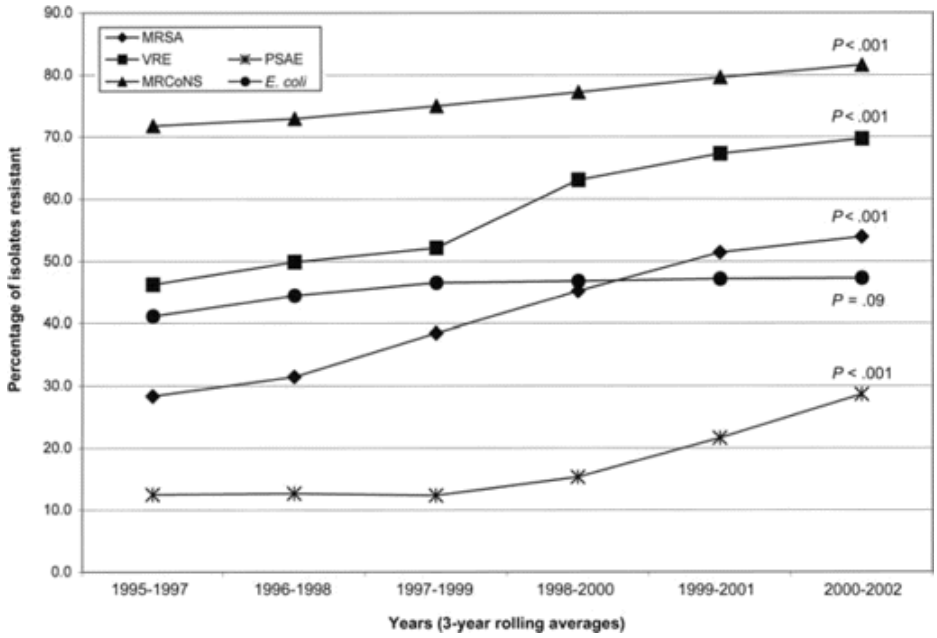


Figure 1-1: Rates of antimicrobial resistance rates over time among Gram positive isolates; methicillin-resistant Staphylococcus aureus (MRSA), methicillin-resistant coagulase-negative staphylococci, vancomycin-resistant Enterococcus faecium (VRE), ampicillin-resistant Escherichia coli (E. coli), and ceftazidime-resistant Pseudomonas aeruginosa recovered in a series of 24,179 cases of hospital; acquired bloodstream infection;[3] *Figure reprinted with permission, copyright Oxford University Press*

Antimicrobial peptides (AMPs) are one class of natural products that are seeing an increase in interest from pharmaceutical research and development. Many peptides are found to have potent antibacterial activity and a low propensity to select for resistance but they are held back from clinical use by their poor drug-like properties. New developments in peptide chemistry and design are enabling a new generation of peptide therapeutics that can be selective and non-toxic, orally bioavailable,[8] even multifunctional[9] or compatible in combination therapies.[9]

1.1 Antimicrobial Peptides

A huge wealth of AMPs exist in nature; they are produced by virtually all classes of organism, occurring as part of innate and adaptive immune systems of prokaryotes (bacteriocins) and eukaryotes. In multicellular organisms, they are largely found in epithelial surfaces where microbes are likely to be encountered but they are also involved

in more complex, self defence mechanisms to protect against invading pathogens. Ganz defined AMPs as "polypeptide antimicrobial substances, encoded by genes and synthesized by ribosomes, with fewer than 100 amino acid residues".[10]

AMPs are commonly found to have broad spectrum activities, inhibiting or killing a large number of Gram positive and Gram negative bacteria as well as a whole host of other activities such as antileishmanial,[11] antitumour,[11] antifungal[12] and antiendotoxin.[13] The majority of peptides reported tend to have net cationic charges but there are a number of anionic AMPs[14] and neutral peptides reported.[15] Many AMPs have been found to target bacterial membranes and whilst some are found to have disordered solution structures it is well established that on contact with cell or model membranes they form amphipathic secondary structures, commonly alpha helices or beta sheets, where charged and hydrophobic residues line up on opposite sides of the structure allowing the peptides to interact with membranes through both electrostatic and hydrophobic interactions. This forms the basis of their mechanism of action which is largely thought to be disruption of cell membranes through pore formation but it is increasingly understood that many also, or only, have intracellular targets.[16] Many also have immunomodulatory effects and for host defence peptides this can be their main biological activity.[17]

Peptide Family	Origin	Reported Biological Activities
Magainins[18]	Frog	Antibacterial, antitumour[19]
Mastoparans[20]	Wasp	Antibacterial,[21] mast cell degranulation,[20] stimulation of insulin release[22]
β-Defensins[23]	Mammal	Antibacterial,[23] immune modulatory activity,[23]
Protegrins[24]	Mammal	Antibacterial,[24] immune modulatory activity[24]
Thionins[23]	Plant	Antibacterial,[23] antifungal,[23] toxicity towards mammalian cells[23]

Table 1-1: Examples of different families of AMPs and some of their reported activities

AMPs are found to have a very low propensity to select for resistance but there are some reported mechanisms: the production of proteases,[9] increased number of exporter pumps[25] and altering the cell membrane to reduce the net negative charge.[26]

Selectivity among AMPs is usually determined *in vitro* by comparing haemolysis assays with minimum inhibitory concentration (MIC) assays and is found to vary widely. The mechanisms by which it is exerted are not fully understood but, where it is present, selectivity is attributed to the increased affinity for the more negatively charged bacteria

cell membrane and the presence of cholesterol in mammalian cells contributing stability to the cell membrane.[27] Haemolysis assays can be poor indicators of *in vivo* toxicity and this is particularly true for AMPs which can have immunomodulatory activity. The toxicity of AMPs for systemic application is also poorly understood which is reflected in the lack of *in vivo* studies in the literature. Most AMPs currently in clinical or pre-clinical development are topical creams or gels for the treatment of skin infections,[28–30] inhalational formulations to treat respiratory tract infections[31] or intended for the treatment of infections of the gastrointestinal tract.[32,33]

1.1.1 Mode of Action for Membrane Active AMPs

Peptides that target bacterial cell membranes must initially pass through the cell wall. It is well established that they then bind parallel to the lipid bilayer and, at low concentrations, embed into the lipid head group region, this is known as the surface state. Although this is largely referred to as an inactive state, thinning of the membrane is observed as the peptides increase the surface area of the head group region, this is often reversed on formation of pores.[34] As the concentration of bound peptide increases, the peptides will be increasingly observed as perpendicular to the bilayer as they insert into the membrane. Insertion and the extent of membrane disruption is dependent on both the properties of the peptide and the lipid content of the membrane. There are three main models to explain how membrane disruption is believed to work: 'barrel-stave', 'carpet' and 'toroidal-pore' (*Figure 1-2 to Figure 1-4*)

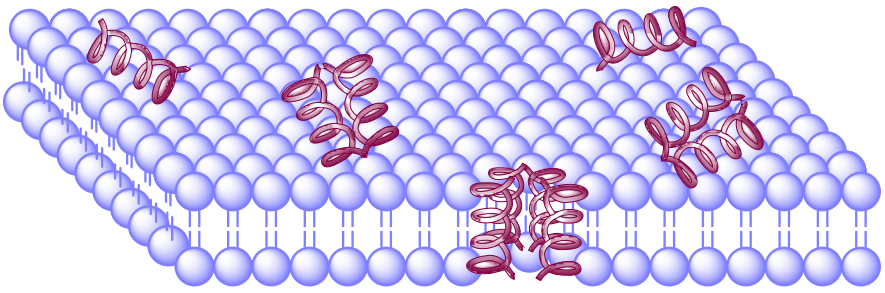


Figure 1-2: Barrel-stave pore formation in a lipid bilayer

The barrel-stave model was the first model developed for membrane disruption when it was used to describe the ion channels alamethicin formed in lipid bilayers[35] and it is still believed to be unique to alamethicin. A variety of studies have shown that alamethicin helices associate to form a barrel-like bundle in the membrane with the hydrophobic

peptide regions aligning to face the internal lipid core of the membrane and the hydrophilic peptide regions facing inwards forming the interior of the pore.[36,37]

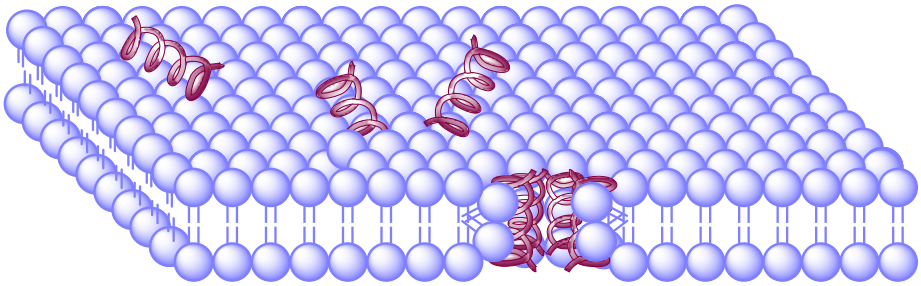


Figure 1-3: Toroidal-pore mechanism in a lipid bilayer

The toroidal pore mechanism (*Figure 1-3*) occurs when peptide helices insert into the bilayer and cause curvature in the lipid monolayers so that the interior of the pore is lined by the peptide and the head group of the lipid.

Unlike the other two models, the carpet model (*Figure 1-4*) proposes that peptides do not insert themselves into the lipid core of the membrane.[38] It is also understood that this mechanism does not require peptides to adopt a specific secondary structure on interaction with the membrane or to aggregate with one another.[39] Once a high local concentration of peptide is reached toroidal pores are formed and the membrane is disrupted in a detergent like manner and can eventually disintegrate completely.[40] Some researchers consider this to be the same as toroidal pore formation but at higher peptide concentrations.

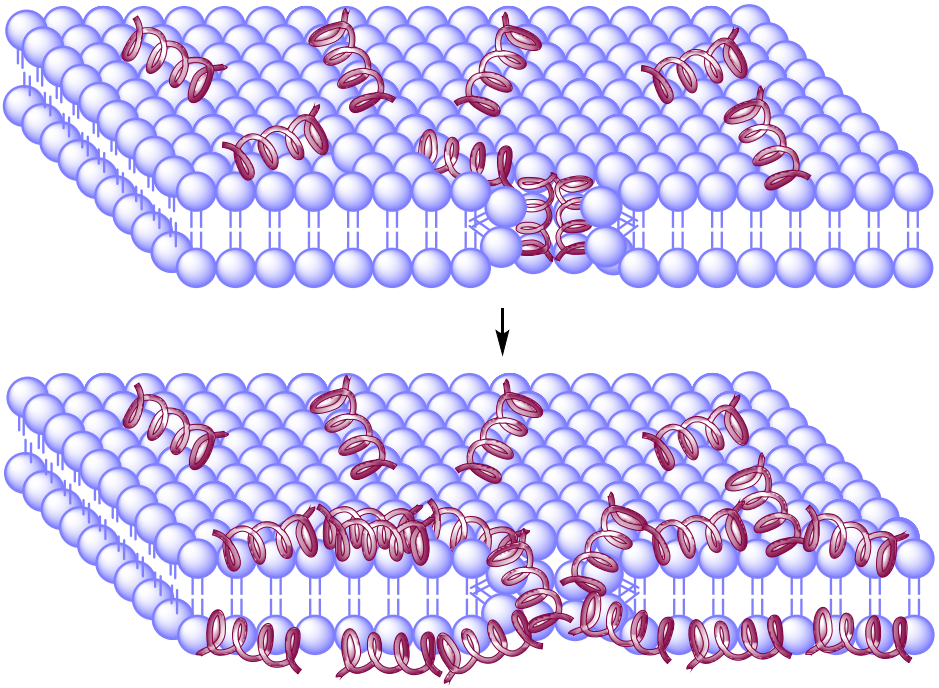


Figure 1-4: Carpet model in a lipid bilayer

AMPs are fairly easy to synthesise and there has been a wealth of structure activity relationship studies carried out looking at the effect of charge,[41] length of peptide,[42] and amphipathicity.[43] These are not straightforward to study as changing one parameter will usually impact the others. For more in depth discussion of these studies see reviews by Tossi *et al*.[44] and Huang *et al*.[45]

1.1.2 Pexiganan

Pexiganan was developed through structure activity relationship studies from magainin, a well-studied model AMP. Magainins were first isolated from the skin of an African claw frog by M. Zasloff who noticed that after non sterile surgery the frogs rarely suffered from bacterial infections.[18] Extracts from the skin were tested for antimicrobial activity against *E. coli* and the fractions that showed activity were further purified. Reverse phase HPLC showed there were five main components with antibacterial activity. The best two of these were then sequenced and further screened for antimicrobial activity and they showed good activity against both Gram-negative and Gram-positive bacteria, fungi, and protozoa (**1** and **2**, *Figure 1-5*). They were named "magainins" which was derived from the Hebrew word "magain" meaning "shield" due to their ability to protect from infection.

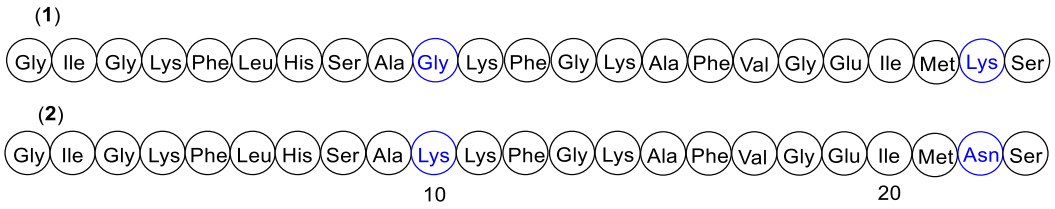


*Figure 1-5: Magainin 1 (**1**) and 2 (**2**) isolated by Zasloff, differences highlighted in blue*

It was thought that the magainin peptides could be membrane disrupting peptides due to the structural similarity to other peptides already shown to disrupt membranes such as mellitin. To probe their activity further, Zasloff went on to use to synthesise the characterised peptides (**1**), (**2**) and, in addition, a C-terminus truncated analogue of (**2**) that had also been identified as one of the five main components of skin extracts (**3**, *Figure 1-6*).[46] A successive series of N-terminus truncated peptides were also synthesised (**4**-**9**, *Figure 1-6*).

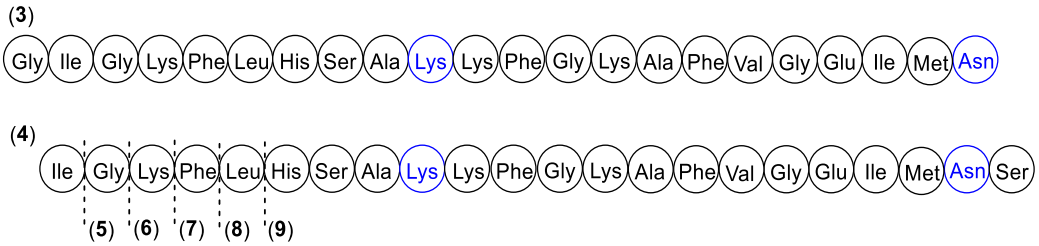


Figure 1-6: Truncated analogues of magainin synthesised by Zasloff et al.[46]

Magainin 1 (**1**), 2 (**2**) and the C-terminus truncated analogue (**3**) were tested against a range of Gram positive and Gram negative bacteria for antibacterial activity and were shown to have broad spectrum activity (*Table 1-2*). MIC data was not obtained for the N-terminus truncated analogues but solutions of peptide (50 µg in 10 µl water) were spotted on agarose plates spread with *E. coli*. The first two truncations (**4**-**5**) were well tolerated, showing large "halos" of growth inhibition of *E. coli* but removing four or more amino acids (**6** and **7**) showed little inhibition of growth. Peptides with truncations of five or six (**8** and **9**) amino acids showed no activity against *E. coli* using this assay. It was suggested that the decrease in activity was due to the peptides no longer being able to span the bacterial membrane as an α-helical structure. In the same study, a protozoan, *Paramacium caudatum,* was treated with magainin 2 and it was observed that this caused osmotic swelling of the cell which resulted in the cell bursting. Leakage from model membranes

encapsulating fluorescent markers[47] and the disruption of electrochemical ion gradients across cell membranes[48] had also been observed.

Organism (ATCC no.)	MIC µg/mL		
	Magainin 1 (1)	**Magainin 2 (2)**	**Truncated Magainin (3)**
Escherichia coli D31	1-7	1-7	1-7
Streptococcus pyogenes (19615)	7-35	1-7	7-35
Enterobacter cloacae (23355)	7-35	1-7	7-35
Staphylococcus epidermidis (12228)	>100	7-35	35-70
Staphylococcus aureus (25923)	>100	35-70	>100

Table 1-2: Selection of MIC values obtained by Zasloff et al.[46]

Solid state ^{15}N NMR on model membranes of phospholipids showed magainin 2 (**2**) existed as a helical peptide in membrane environments and lay parallel to the plane of the membrane.[49] This was supported by earlier work involving fluorescence quenching experiments carried out with phenylalanine containing analogues of magainin[50] and also Monte Carlo simulations.[51] Wade *et al.*[52] showed that the all D-enantiomer of the natural L-magainin had equivalent antibacterial activity and produced the same current in conductivity measurements in model membranes, which indicated the target was not a protein or enzymatic target. These experiments only accounted for interactions with the head groups of the membrane and did not show any significant interaction with the alkyl chain region.

Ludtke and co-workers were the first to publish evidence that suggested at high concentrations (around a 1:30 peptide to lipid molar ratio) magainin inserts itself into model membranes.[53] Using oriented circular dichroism (OCD) measurements (a technique used to assess orientation of a peptide with respect to a lipid bilayer as well as its secondary structure)[54] they saw changes in OCD spectra that indicated a shift in orientation of magainin from parallel to perpendicular to the plane of the lipid membrane. The concentration at which the shift occurred corresponded well with the concentration required for lysis in liposome leakage experiments, also around 1:30.[55]

Magainin peptides were later shown to have anti-cancer activity, causing lysis of tumour cells at concentrations non-toxic to peripheral blood cells.[56] Cancer cell membranes are more fluid and more negatively charged than mammalian cells making them a prime target for membrane disrupting AMPs.

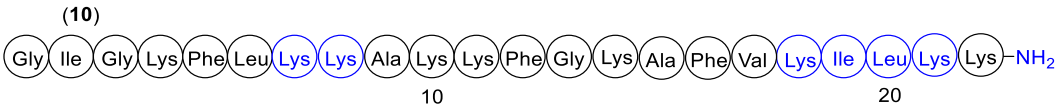


*Figure 1-7: Sequence of pexiganan (**10**); differences to magainin 2 (**2**) highlighted in blue*

Several structure activity relationship studies were carried out on magainin but it was Zasloff and co-workers who founded a small biotech company, Magainin Pharmaceuticals, and developed the most successful analogue, pexiganan (**10**, *Figure 1-7*). This analogue was amidated at the C-terminus and contained more basic lysine residues than either magainin 1 (**1**) or 2 (**2**). Extensive *in vitro* screenings showed pexiganan to have broad spectrum activity against both Gram positive and Gram negative bacteria[57] including a number of antibiotic resistant strains, showing no signs of cross resistance and also no signs of selecting for resistance. Pexiganan (**10**) was also shown to have equivalent *in vitro* bactericidal activity to ofloxacin,[58] a commonly used antibiotic. In 1999 it underwent clinical trials as a 1% cream formulation for the topical treatment for diabetic foot ulcers.[59] The trials determined pexiganan was no more effective than already approved treatments for diabetic foot ulcers and so failed to obtain approval by the US Food and Drug Administration (FDA). Now owned by Dipexium Pharmaceuticals Inc., pexiganan has recently gone through a second round of clinical trials but was again deemed no more effective than existing treatments.

Similar to magainin, solid state NMR has shown pexiganan exists in a disordered structure in solution but on binding to both lipid bilayers and detergent micelles it forms a defined secondary structure, a dimeric antiparallel α-helical coiled-coil.[60,61] NMR studies have also shown that pexiganan is most likely to form pores in a toroidal like mechanism.[62] This has been supported by a number of other studies including the use of differential scanning calorimetry to show the induction of positive curvature strain on the lipid bilayer when pexiganan is bound.[62]

1.1.3 Mastoparan

Mastoparan (**11**, *Figure 1-8)* was first isolated in 1979 by Y. Hirai whilst analysing the complex mixture of bioactive compounds in wasp venom and it was shown to cause degranulation of mast cells.[20] It was later shown to have anti-microbial activity and increased the permeability of cell membranes to potassium ions in both Gram negative and

Gram positive bacteria.[63] Haemolytic activity was also observed but required much higher concentrations of peptide. Since 1979 many more analogues of mastoparan have been isolated from different species of wasps[64,65,66] and many more generated through synthetic manipulation of the peptides.[67] Mastoparan has been found to have a number of other biological activities including stimulating insulin production in pancreatic islet cells[22] and myotoxic effects in mice skeletal muscle.[68]

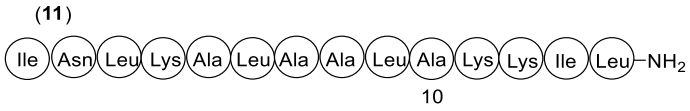


*Figure 1-8: Sequence of the first mastoparan peptide isolated (**11**)*

Mastoparans are generally found to be tetradecapeptide amides and are now characterised by their ability to cause mast cell degranulation. Mast cells are leukocytes that are part of the immune and neuroimmune systems, playing a key role in participating in early recognition of pathogens and inflammation.[69] They contain granules rich in immune response mediators such as histamine, heparin and proteases. It is thought mastoparans trigger exocytosis of these granules through interaction with G-protein receptors.[70] Once released these go on to modulate the host immune response in a variety of ways for example by releasing their own AMPs and the release of mediators that go on to promote neutrophil recruitment.

Experiments by Katsu *et al.*[71] suggested there was a correlation between the ability of mastoparan peptides to release histamine, cell permeability and their antimicrobial activity. As well as interacting with G-protein receptors mastoparans were also membrane active towards mast cells; this was attributed to the increase in negative charge of the cytoplasmic membrane arising from histamine release. This study again confirmed that much higher concentrations of peptide (> 80 μM) were required for a haemolytic effect.

Eliciting an immune response is generally considered to be an unwanted side effect of AMPs as the response is hard to study and predict *in vitro* and *in vivo* and can result in adverse effects in clinical trials. However, a situation in which an AMP such as mastoparan could also elicit a controlled immune response would result in a very effective therapeutic therapy against bacterial infections. Much more work on understanding the immune responses that AMPs elicit needs to occur before such therapies are a realistic prospect.

For now, the promiscuity of mastoparan for other biological targets limits its therapeutic potential especially if considering its use as a systemic drug.

One analogue of mastoparan that shows promising therapeutic potential and greater selectivity than other analogues is Polybia-MP1 (**12**). It was first isolated by B. Souza *et al*.[21] in 2005 from the venom of the wasp *Polybia paulista* alongside another less potent analogue, Polybia-CP (**13**).

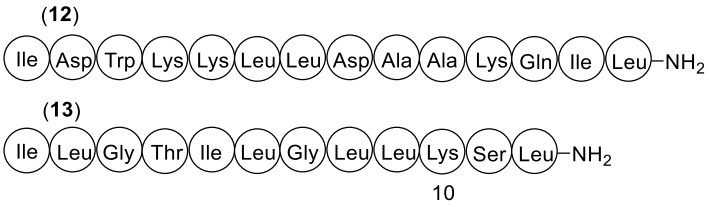


*Figure 1-9: Sequences of Polybia-MP1 (**12**) and Polybia-CP (**13**)*

Polybia-MP1 (**12**) was shown to have antimicrobial activity against both Gram positive and Gram negative bacteria but did not cause haemolysis to rat erythrocytes and whilst mast cell degranulation activity was recorded it required significantly higher concentrations of peptide than relevant for physiological concentrations.[21]

Further studies involving molecular dynamics simulations, circular dichroism and dye leakage assays using both anionic and zwitterionic vesicles were carried out to investigate the selectivity of Polybia-MP1 (**12**).[72] It was suggested that the selectivity was a result of the distribution of the charged and polar residues along the peptide helix and the resulting orientation of solvent molecules. The presence and position of aspartic acid residues appeared to be especially important, a single substitution of Asp2 residue for Asn2, led to a haemolytic analogue. It was suggested that the partial neutralisation of the cationic residues that aspartic acid provides causes a smaller disturbance of the head group region of zwitterionic lipids of erythrocytes and impacts the membrane destabilising ability of the peptide.

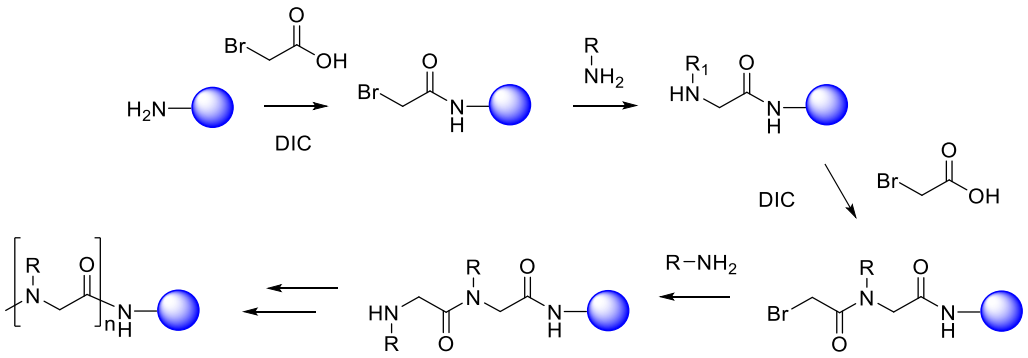
1.1.4 Peptoids

Peptoids were developed as a tool for drug discovery; they provided a way to rapidly and cheaply generate vast libraries of small molecules for screening for biological activity. A small team of scientists at a biotechnology company, Chiron Corp., wanted to mimic the

way nature uses a finite selection of small building blocks to create diverse collections of biopolymers.[73] This approach was also attractive as a limited number of building blocks would only need a limited number of reactions to put them together. Once that methodology was in place a small team of chemists could generate vast libraries of compounds. The researchers at Chiron Corp. proposed the five most desirable attributes of these systems:

"(i) the monomers should be straightforward to synthesize in large amounts, (ii) the monomers should have a wide variety of functional groups presented as side chains off of the oligomeric backbone, (iii) the linking chemistry should be high yielding and amenable to automation, (iv) the linkage should be resistant to hydrolytic enzymes, and (v) the monomers should be achiral."[74]

With these criteria in mind, the team developed a set of peptide mimics with oligomeric N-substituted glycines where the alpha carbon side chain of an amino acid was shifted onto the amide nitrogen. In comparison to peptides these oligomers lacked an amide hydrogen bond donor and chirality at the alpha carbon. The biggest advantage over peptides was that these oligomers, or peptoids, were no longer susceptible to proteases as they no longer contain a peptide amide bond. In order to avoid having to prepare a set of N-substituted glycine monomers (the peptoid equivalent of amino acids) Zuckermann and co-workers developed a sub monomer approach whereby the peptoid chain can be built up with alternating condensation of copolymers of a haloacetic acid and a primary amine (*Scheme 1-1*).[75]



Scheme 1-1: Peptoid synthesis for a sub-monomer approach

Peptoids have now been shown to be adequate peptide mimics and have wide range of biologically relevant activities including anti-leishmanial,[76] activity against bio-films,[77]

mimics of peptide lung surfactants,[78] mimicking protein secondary structures,[79] inhibiting protein-protein interactions,[80] and cell penetrating abilities.[81]

Alongside the development of pure peptoid structures, peptoid building blocks can be incorporated easily in a peptide sequence[82] to give peptoid-peptide hybrids or "peptomers". AMPs are ideal candidates for peptoid substitutions as one of their main disadvantages is their susceptibility to degradation *in vivo* by proteases and this can be minimised with increasing peptoid character. As previously discussed, the amphipathic nature of AMPs is what drives their activity, this is determined by the distribution of hydrophobic and cationic residues and this distribution can be relatively easily replicated with the relevant peptoid monomers.

Building on work that had shown that both monomers with bulky alpha-chiral aromatic side chains[83] and those with bulky alpha-chiral aliphatic side chains[84] give rise to peptoids with stable helical structures, Patch and Barron synthesised a set of peptoids with helices that were predicted to be similar to that of magainin 2 (*Figure 1-10*).[85] The mimics were of 12 and 17 monomers in length as this gave predicted helix lengths in line with the α-helical conformation of magainin-2. The most promising of the mimics (**15**) and (**17**) gave potent antibacterial activity against both Gram-positive and Gram-negative bacteria with selectivity, as determined by haemolysis assays, comparable to that of magainin (

Table 1-3).

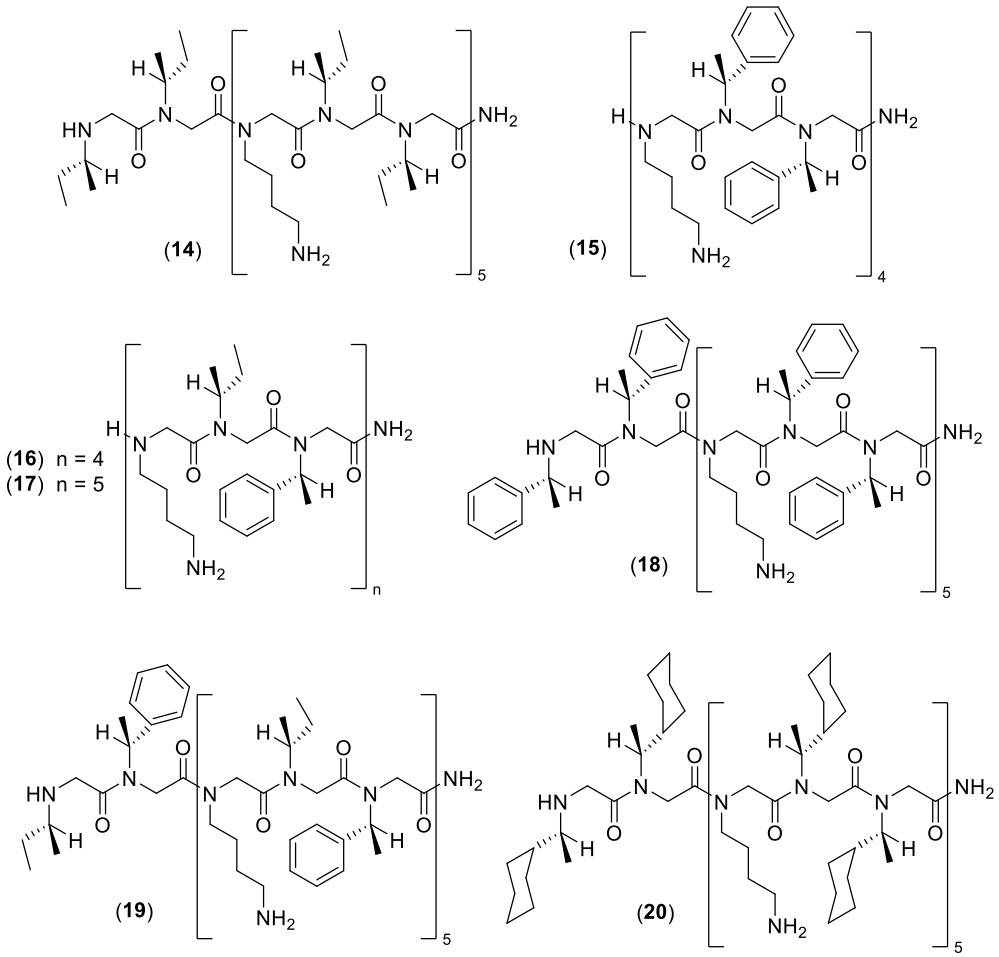


Figure 1-10: Peptoid mimics of magainin 2 amide

Peptoid	*E. coli* MIC (μM)	*B. subtilis* MIC (μM)	Haemolysis at *E. coli* MIC (%)
14	>100	>100 (>200)	0
16	49 ± 8.3	7.8 ± 1.3	0
17	9.9 ± 2.5	4.4 ± 0.8	0
19	19 ± 4.9	1.4 ± 0.2	1.2
15	5.4 ± 0.9	0.82 ± 0.14	1.4
18	7.7 ± 1.3	1.2 ± 0.2	51
20	>75	>75	100[a]

Table 1-3: Peptoid antibacterial and haemolytic activities obtained by Barron[85]
Estimated uncertainties of MIC data indicated; [a]reported at 75 μM[85]

Further structure activity relationship studies were carried out by the same group with the most promising analogue (**15**). The effects of chirality, length, charge, amphipathicity and hydrophobicity on potency and selectivity were investigated.[86] The group were able to produce 6 variants that had improved selectivity over $(NLysNspeNspe)_4$ (**15**), determined by comparing the haemolytic data with MIC data (

Table 1-4). The most selective was a reduced hydrophobicity variant, 1-Pro6 (**26**), where Nspe at position 6 was replaced with L-proline. This analogue was shown to have similar antibacterial activity to pexiganan (**10**) but was not quite as selective as the AMP. However, in a cytotoxicity assay using lung epithelial cells the peptoid (**26**) performed 10 x better than the AMP with a 10% metabolic inhibitory dose (ID_{10}) of 22.8 µM. The group concluded that the activity of peptoids depends on moderate hydrophobicity and net cationic charge and their haemolytic activity relies mostly on high hydrophobicity and strong amphipathic structure in a very similar fashion to AMPs.

Sequence	*E. coli* MIC (µM)	*B. subtilis* MIC (µM)	Selectivity ratio (SR)[a]
H-(NLys-Nspe-Nspe)$_4$-NH_2 (**15**)	3.5	0.88	6.0
H-(NLys-Nspe-Nspe)$_2$-NH_2 (**21**)	27	27	>8.1
H-(NLys-Nspe-Nspe)$_3$-NH_2 (**22**)	9.1	1.2	>16
H-(NLys-Nsmb-Nspe)$_4$-NH_2 (**23**)	7.4	0.95	>16
H-(NLys-Nssb-Nspe-NLys-Nssb-Nsna)$_2$-NH_2 (**24**)	7.2	0.93	7.6
H-(NLys-Nspe-Nspe-NLys-Nspe-NHis)$_2$-NH_2 (**25**)	3.5	6.9	>31
H-NLys-Nspe-Nspe-NLys-Nspe-L-Pro(NLys-Nspe-Nspe)$_2$-NH_2 (**26**)	3.1	1.6	20
GIGKFLKKAKKFGKAFVKILKK-NH_2 (**10**)	3.1	1.6	24

*Table 1-4: Antibacterial and haemolytic activities of peptoids (**15**, **21**-**26**) and pexiganan (**10**) obtained by Barron*[86]
[a]Selectivity ratio, SR = (10% haemolytic dose)/(E. coli MIC)

Acknowledging that haemolytic assays are not always good predictors for *in vivo* toxicity, Bolt *et al*.[87] have recently generated a large peptoid library and tested for toxicity using two mammalian cell lines. Like Barron and co-workers, they found that the most toxic peptoids tended to have higher hydrophobicity and, interestingly, sequences with reduced frequency of chiral monomers, compared to the standard NxNspeNspe motif, showed reduced toxicity. They go on to suggest that the selectivity they observe indicates that cell membrane disruption is not the only mechanism at work.

1.2 Lantibiotics

Lantibiotics are a subset of AMPs that have seen an increase in attention from pharmaceutical research and development over the last decade. They are ribosomally synthesised by a wide range of bacteria and form a substantial class of AMPs.[88] Their structural complexity is notable, owing in part to the extensive range of post-translational

modifications they undergo.[89] The name originates from the inclusion of one or more of the uncommon amino acids, lanthionine (Lan) (**29**) and methyllanthionine (MeLan) (**30**). These thioether analogues of cystine are the product of enzymatically controlled Michael addition to dehydroalanine (Dha) (**31**) and dehydrobutyrine (Dhb) (**32**) which are derived from the dehydration of serine and threonine respectively. These modifications give rise to peptides with multiple, often overlapping, cyclic motifs, some examples of these peptides can be seen in *Figure 1-11*.

Lantibiotics are broadly divided into three classes, Type I, II and III, defined by their mode of action and the type of enzymes involved in their biosynthetic pathways. Type I lantibiotics such as nisin (**27**) and epidermin (**28**), are flexible, elongated molecules whose primary mode of action is binding and sequestering a cell wall precursor, lipid II, and forming pores in the bacterial plasma membrane. Type II Lantibiotics, such as cinnamycin (**33**) are more rigid and globular in shape and can bind lipid II but are less likely to have pore forming abilities. Type III form the smallest class of lantibiotics and are found to have long, hydrophobic sequences but little antimicrobial activity. Lantibiotics are also sometimes produced as two-component systems where an organism produces two separate peptides that work synergistically together such as lacticin 3147 (**35** and **36**) in which lacticin A1 (**35**) binds to lipid II and lacticin A2 (**36**) forms pores in membranes, but only in the presence of lacticin A1 (**35**).[90]

Due to the lipid II dependant nature of their mode of action lantibiotics are primarily active against Gram positive bacteria as the thick outer membrane of lipopolysaccharides of Gram negative bacteria prevents the peptides from reaching lipid II. High concentrations of peptide or the addition of membrane destabilising agents can improve activity against Gram negative bacteria.[91]

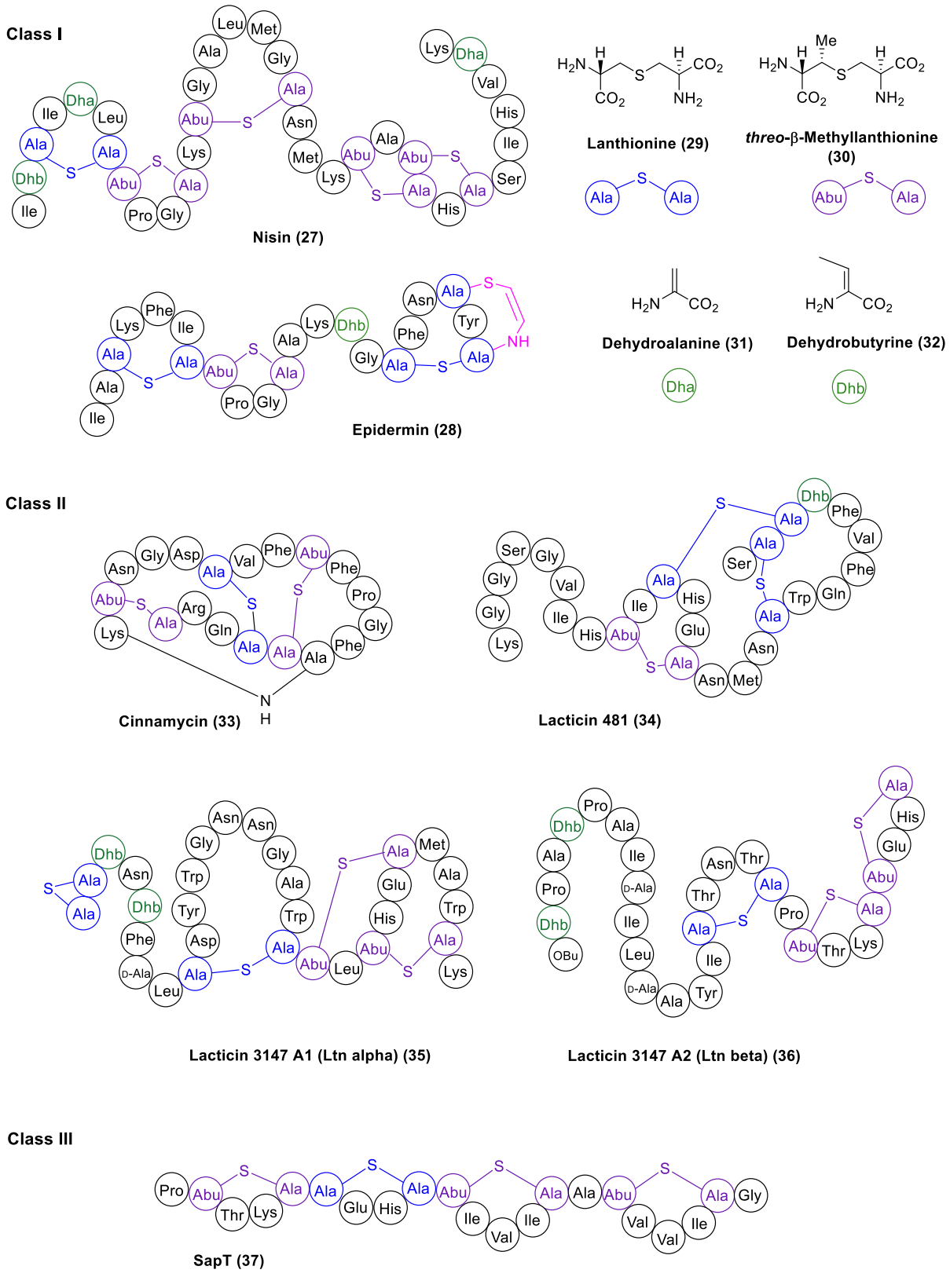


Figure 1-11: Examples of lantibiotics

A significant number of lantibiotics are reported to have excellent antibacterial activity, notably against multi-drug resistant strains of bacteria, but their development as drugs has been held back by poor drug-likeness and a lack of synthetic methods available to generate or modify their complex structures.

Poor drug-likeness can be attributed to the chemical instability of lantibiotics: sulphur-containing lanthionine residues are prone to oxidation and unreacted dehydro residues can be easily cleaved in basic conditions, attacked by nucleophiles or undergo hydrolysis in strongly acidic conditions.[92,93] Solubility can also be a problem, notably, nisin is poorly soluble at neutral or high pH[94] and whilst the thioether bridges of lantibiotics provide some protection to proteolysis and thermal degradation[95] they are still prone to enzymatic degradation in the gastrointestinal tract and in the bloodstream.[96] This means lantibiotics are unlikely to be orally bioavailable.

However, as the pressure for new antimicrobials mounts, there are now a small handful of lantibiotics undergoing clinical trials. NVB302 (**38**, *Figure 1-12*), a C-terminally modified derivative of a type II lantibiotic, actagardine, is currently being developed by Novacta Biosytems and has completed Phase I clinical trials for the treatment of *Clostridium difficile* infections.[97]

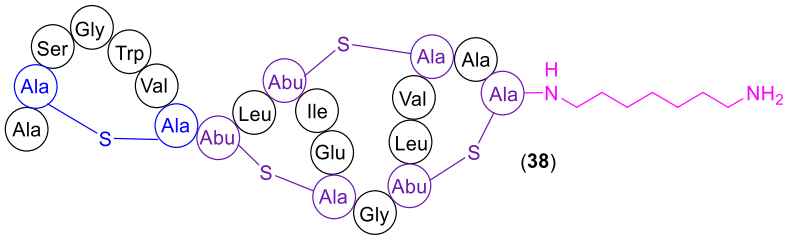


*Figure 1-12: Structure of NVB302 (**38**)*

1.2.1 Nisin

Nisin (**27**) is the most widely studied member of the lantibiotics. It has broad spectrum antimicrobial activity, notably against MRSA and VRE.[98] Nisin also happens to have good activity against food spoilage bacteria which has been put to use in its role as a food preservative in the dairy industry.[99] It is produced by lactic acid bacterium *Lactococcus lactis*[100] and a modified strain of this is used industrially in a neutral fermentation process to produce enough peptide for the dairy industry.[101] When ingested orally nisin is

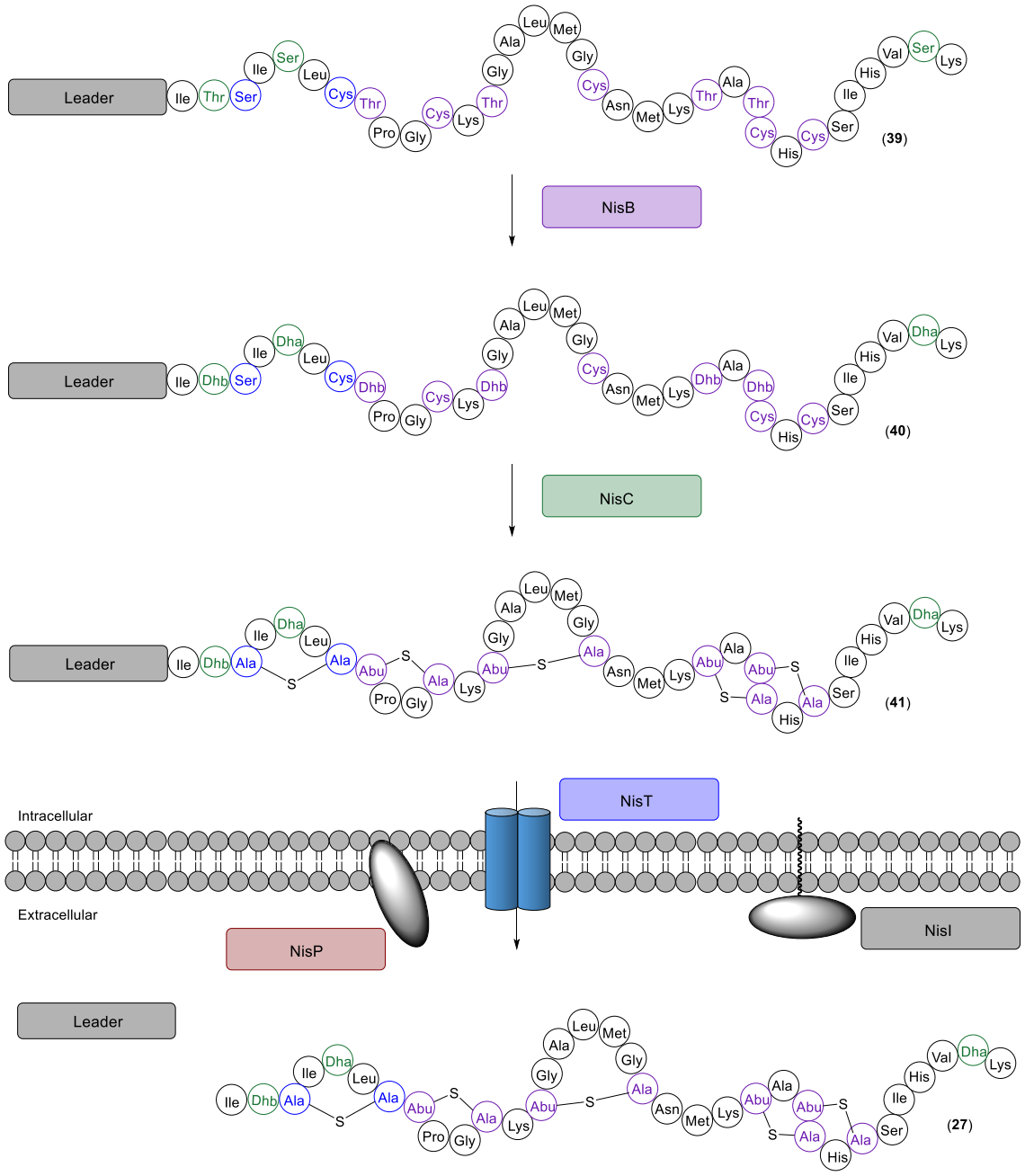
considered non-toxic as it is broken down in the acidic conditions of the stomach.[96] One of the most significant features of nisin is that despite widespread use as a preservative over a period of decades no significant incidences of resistance have been reported.

There are now eight natural variants of nisin reported. The first two isolated, and the most studied are nisin A and nisin Z. These differ by a single amino acid substitution at position 27; in nisin A this is histidine and for nisin Z it is asparagine. Antimicrobial activity is not affected by the substitution but nisin Z has been found to have better diffusion and solubility properties.[102] Despite the fact that nisin is the most extensively studied lantibiotic its mode of action is not fully understood at a molecular level.

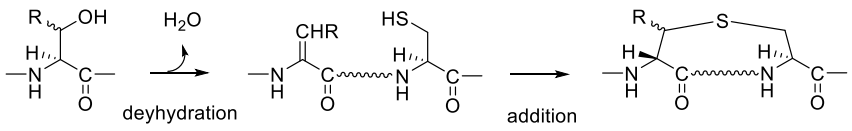
1.2.2 Biosynthesis of Nisin

Biosynthesis begins with the NisA gene which encodes the 57 amino acid straight chain "prepeptide" of nisin (**39**, *Scheme 1-2*). This is ribosomally synthesised with an N-terminal "leader" peptide section which serves two main functions: guiding and activating the lanthionine modification enzymes and keeping the core peptide inactive during maturation.[103] After ribosomal synthesis, the NisB dehydratase enzyme selectively catalyses the dehydration of the necessary serine and threonine residues (*Scheme 1-3*). This is followed by the regio- and stereo-specific Michael addition of the cysteine residues to the newly formed Dha and Dhb residues catalysed by the NisC cyclase enzyme to give the five rings of nisin (**41**).[104] For nisin this reaction produces only DL-lanthionine and methyllanthionine which is the most commonly reported stereochemistry for lanthionine residues but some exceptions have been found in other lantibiotics.[105]

After cyclisation NisP protease cleaves the leader peptide to give nisin A. NisT is also involved in the biosynthesis as an ATP-binding cassette transporter responsible for the secretion of the final product (**27**).[106]



Scheme 1-2: Biosynthetic pathway of nisin



Scheme 1-3: Dehydration of serine (R= H) and threonine (R = Me) residues to Dha and Dhb followed by cyclisation

The antibacterial activity of nisin means that a system of immunity needs to work alongside the biosynthetic pathway to protect the producing strain of bacteria from the effects of its own product and in turn, ensure a good rate of production of nisin. Features of immunity were first uncovered in work characterising the biosynthetic pathway of nisin[107,108] and it was further probed by Ra and co-workers who used a "knockout" technique on the genes believed to be responsible for immunity.[109] Another key study was carried out by Stein and co-workers who engineered a nisin-sensitive strain of *Bacillus subtilis* with different combinations of nisin immunity genes and measured the impact on cell viability.[110] It is understood that immunity is conferred by a combination of two systems: a lipoprotein NisI and an ATP binding cassette (ABC) transporter NisFEG. The lipoprotein is believed to be transported to the extracellular side of the cytoplasmic membrane where it can interact and intercept nisin and disrupt pore formation. The ABC transporter is thought to work by expelling nisin molecules from the membrane, before or during pore formation, back into the environment.

Some strains of *L. lactis* that do not produce nisin still confer resistance to it. They have been found to produce an enzyme that breaks down nisin and protects the bacteria.[111] Mechanisms of resistance like this are important to study as they could indicate the way in which resistance could develop if nisin, or other lantibiotics, were used in a clinical setting.

1.2.3 Nisin Mode of Action- Initial Studies

Several early experiments suggested nisin's mode of action involved the destabilisation of cytoplasmic cell membranes. To investigate the structure-activity relationship, van der Hooven *et al.*[112] used NMR spectroscopy and model membranes to study the solution structure of the peptide in the presence and absence of model membranes. Both zwitterionic dodecylphosphocholine (DPC) and ionic sodium dodecyl sulphate (SDS) micelles were used to mimic both mammalian and bacterial membranes respectively. Nisin was shown to have two well-defined domains: an N-terminal domain including the A, B and C rings and a second domain consisting of rings D and E. Both components were shown to be amphipathic where charged and hydrophobic residues line up on opposite sides of the structure allowing the peptide to interact with membranes through both electrostatic and hydrophobic interactions. This is a characteristic of many membrane active peptides. Residues 20-23 were later defined as the hinge region, which was shown to be important for adding a degree of flexibility to the molecule.

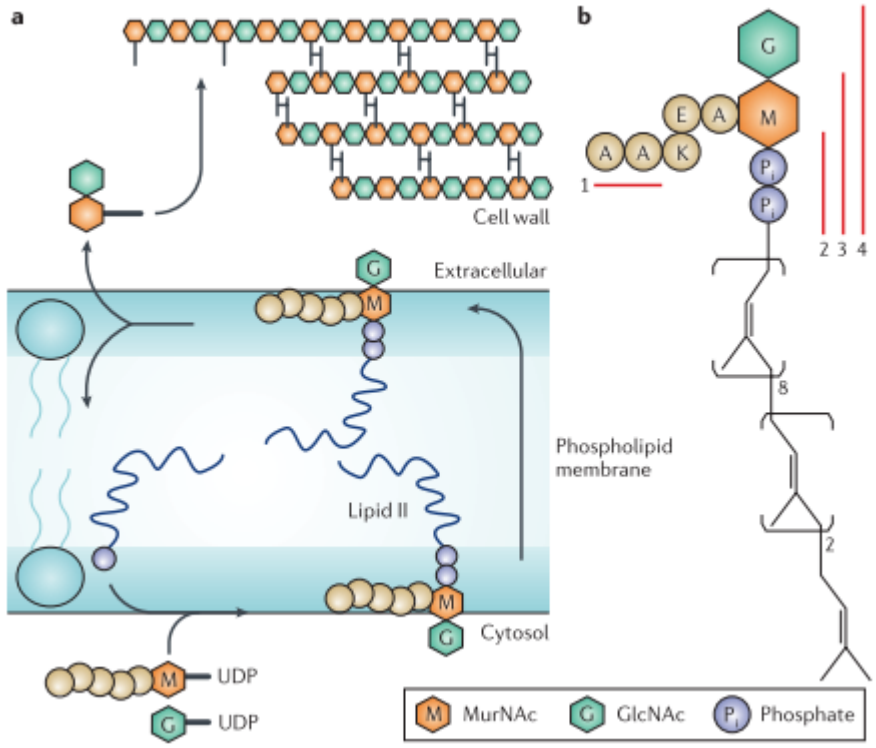
Alongside membrane destabilising activity, nisin had also been shown to inhibit peptidoglycan synthesis *in vitro*[113] but the discrepancy between nisin's activity in model membrane studies which was in the micromolar range, similar to other linear pore forming peptides, and the nanomolar concentrations at which it killed bacteria could not be accounted for. This was until de Kruijff and co-workers observed that extracting lipid content from bacteria and forming model membranes with the resulting lipids also gave better activities for nisin.[114] They hypothesised that nisin could rely on a peptide-based component of the cell wall, lipid II, a cell wall precursor. By using competition assays with vancomycin (a known lipid II ligand) they showed that nisin's activity was indeed likely to rely on lipid II. They went on to incorporate lipid II into model membranes that encapsulated carboxyfluorescein, a fluorescent dye, and could show a strong correlation between lipid II concentration and nisin's ability to cause leakage of the dye. In comparison, magainin a less potent, linear, membrane destabilising peptide showed no dependency on lipid II.

1.2.4 Lipid II as a Target for Antibiotics

All bacterial cell walls are made up of cross-linked, polymeric, amino acid sugars that form a peptidoglycan matrix which is vital in providing the cell wall with its strength and rigidity. The cycle of cell wall synthesis is shown in
Scheme 1-4[115]. Lipid II forms a vital intermediate in the cycle enabling the cell wall subunits to pass from the cytoplasmic side of the plasma membrane, where they are synthesised, to the exterior of the plasma membrane where they are inserted into the growing cell wall. Only small amounts of the precursor, bactoprenyl phosphate, are available within the cell which means only a small amount of lipid II can be generated at any one time. This is compensated for within the cycle by a rapid turnover of around 1-3 cycles per second per prenyl chain[116] and it is this limited availability of lipid II that makes it a weak link in the cell wall synthesis chain and an ideal target for antibiotics. As previously mentioned, lipid II is less accessible in Gram negative bacteria and so they are significantly less susceptible to lipid II targeting antibiotics.

Alongside nisin, there are several other antibiotics that are known to target lipid II. Vancomycin, a glycopeptide, which has been shown to bind to the pentapeptide region of lipid II, specifically to the D-Ala-D-Ala residues, is used clinically to treat MRSA and several other Gram positive bacterial infections.[117] However, unlike nisin, significant resistance to

vancomycin has been reported whereby bacteria have mutated the terminal D-Ala to D-Lac or D-Ser which significantly reduces the binding affinity of vancomycin.[118] Another glycopeptide believed to interact with lipid II is ramoplanin which has undergone phase II clinical trials to treat *C. difficile* infections.[119] The most recent addition to this group of lipid II binding antibiotics is teixobactin which was discovered using novel iChip technology, which allowed for the isolation and culture of bacteria from soil, the majority of which do not grow under standard laboratory conditions.[120] Excitingly, initial studies have suggested teixobactin has a very low propensity to select for resistance and it is believed to form stoichiometric complexes with both lipid II and lipid III. For a more in depth discussion of lipid II binding antibiotics see reviews by Breukink and de Kruijff[115] and more recently by Ng and Chan.[121]



Scheme 1-4: Cell wall synthesis (a) synthesis begins intracellularly with a MurNAc-pentapeptide precursor coupling to bactoprenyl-phosphate, a membrane carrier, to give Lipid I. A second sugar GlcNAc is coupled to lipid I to produce lipid II, which now contains the complete peptidoglycan subunit linked via a pyrophosphate to the membrane-embedded bactoprenol lipid anchor. Lipid II is then translocated to the exterior side of the plasma membrane by an unknown mechanism where it can be incorporated into a polymeric peptidoglycan chain and form the cell wall. (b) Chemical structure of lipid II; red bars indicate the minimal binding sites in Lipid II of glycopeptide antibiotics (1), nisin (2), ramoplanin (3) and mersacidin (4);[115] *figure reprinted with permission, copyright Nature Publishing Group*

1.2.5 Nisin Mode of Action- a Complete Picture?

Although nisin's dependence on lipid II for achieving its maximum potency had been established, the mechanism for the increased activity was still unclear. Using tryptophan mutants of nisin and fluorescence measurements Heusden *et al*.[122] were able to show that lipid II was able to induce a transmembrane configuration of nisin. They suggested that because it would be energetically unfavourable for one molecule of nisin to insert into the hydrophobic membrane it was likely that a pore formed with several nisin molecules creating a water-filled channel in the membrane which the charged residues of nisin would face. The same group went on to show that lipid II acted not just as a recruiting or docking molecule for nisin but actually played a structural role in the pore.[123] Using labelled lipid II molecules and fluorescence measurements they were able to estimate the distance between two lipid II molecules, believed to be situated within a pore, as 18 Å. From this data they proposed a model of the pore that had a possible stoichiometry of five to eight nisin molecules and an identical number of lipid II molecules. This report also suggested that interaction of nisin with lipid II was likely to occur at the pyrophosphate-MurNAc(pentapeptide)-GlcNAc head group of lipid II. Previous using ^{15}N labelled nisin has also suggested that the amide protons of the N-terminus of nisin formed hydrogen bonds with lipid II.[124]

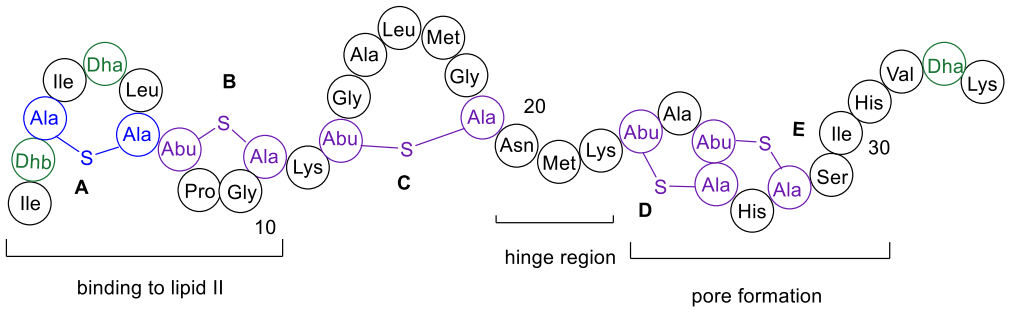


*Figure 1-13: Functional regions of nisin (**27**)*

Hsu *et al.*[125] were able to produce an NMR structure of a 1:1 complex of nisin with a truncated analogue of lipid II (left, *Figure 1-14*). A shorter isoprene tail was used to minimise non-diagnostic signals and to increase water solubility but still provided enough surface for anchoring lipid II into the membrane. Rings AB of nisin were shown to form a cage like structure around the pyrophosphate moiety with hydrogen bonds between the amide groups of the nisin backbone and the pyrophosphate moiety. This was a novel lipid II binding motif and provided an explanation for nisin retaining activity against vancomycin

resistant strains of bacteria as vancomycin binds to the pentapeptide region and resistance arises out of a point mutation of an amino acid. This work provides a much clearer understanding of how nisin binds to lipid II and it also indicates why nisin has such a low propensity to select for resistance as mutation of the highly conserved phosphate group seems unlikely.

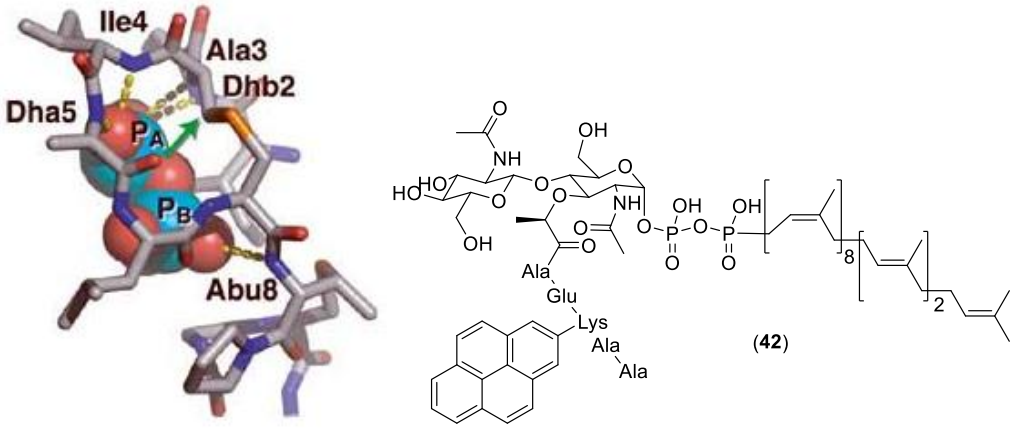
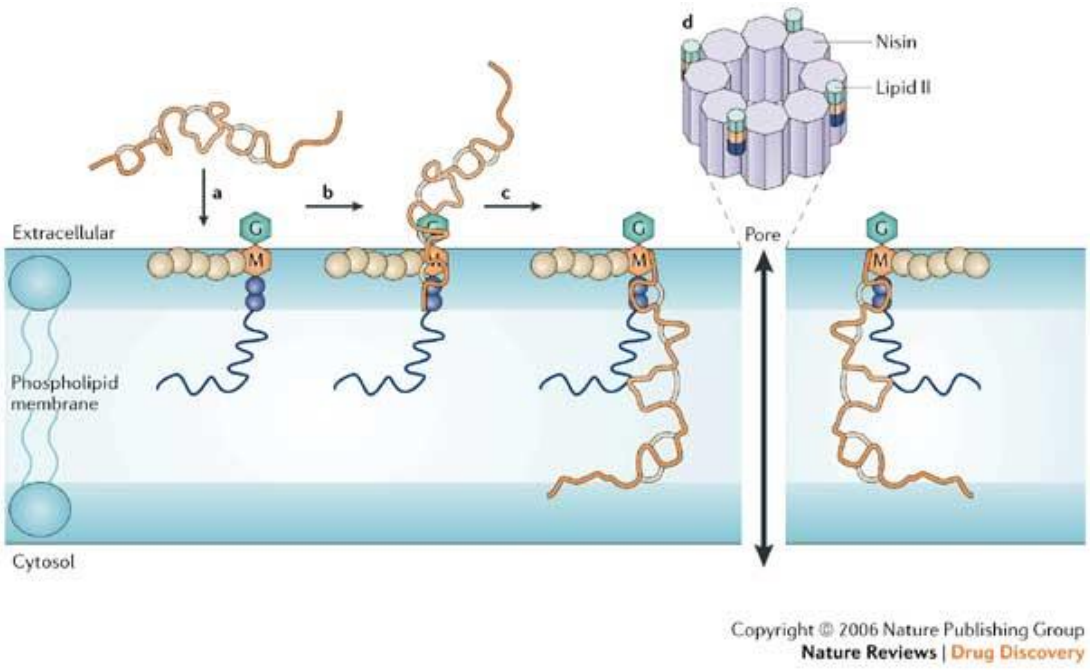


Figure 1-14: Solution structure of the nisin-lipid II complex; backbone of nisin rings AB form a cage-like structure around the pyrophosphate (spheres); hydrogen bonds from the backbone amides that hold the pyrophosphate in place are depicted in yellow; sulphur atoms of the thioether bonds are shown in orange (left);[125] *figure reprinted with permission, copyright Nature Publishing Group; fluorescently labelled lipid II (right,* ***42****) used by Breukink*[126]

Further work by Breukink and co-workers using fluorescently labelled lipid II molecules (right, **42**, *Figure 1-14*) confirmed that the pores in model membranes consist of 8 nisin and 4 lipid II molecules.[126] The stability of these pores was also confirmed as the pores were shown to remain intact even after addition of detergents that solubilized the membranes.

This provides a reasonable model for nisin's pore formation (*Scheme 1-5*) but there are still significant gaps in the current mechanism. It is not known how multiple nisin and lipid II complexes come together and what the forces of attraction and recognition that facilitate this are. The order in which pore formation occurs is not well understood, whether insertion occurs before or after aggregation and the specific role of the hinge region, other than requiring flexibility, is still unclear- is it essential to stable pore formation? Certainly, there is still much more work to be done to complete our understanding of nisin's pore formation. A more robust understanding would facilitate the synthesis of optimal lipid II binding, pore forming therapeutics.

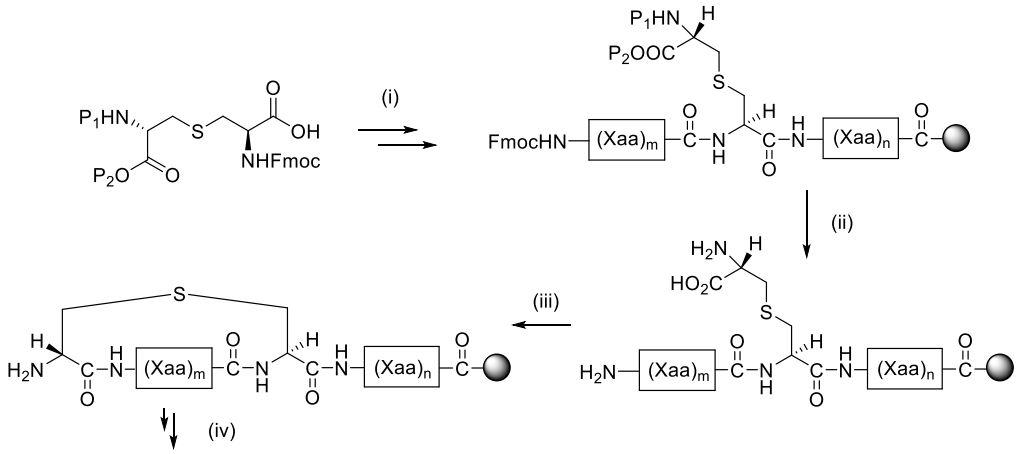


Scheme 1-5: Mechanism of nisin pore formation: (a) nisin reaches the membrane (b) the N-terminus of nisin binds to lipid II forming a 1:1 nisin-lipid II complex (c) these complexes then aggregate and insertion into the membrane takes place whereby several nisin molecules have adopted a trans-membrane orientation with lipid II molecules to form a stable pore structure;[115] *figure reprinted with permission, copyright Nature Publishing Group*

1.3 Synthesising Lantibiotics

There are several synthetic and biosynthetic methods that have been developed over the last few decades to produce meaningful amounts of both wild type and modified analogues of lantibiotics. Each method has its own advantages and disadvantages and to meet the significant challenges of the structural complexity of lantibiotics it's likely that a wide spectrum of tools will be required.

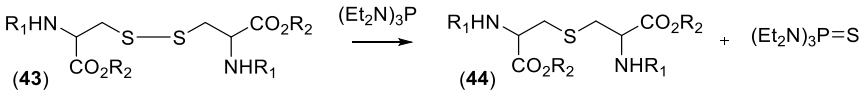
Chemical methods of synthesising lantibiotics largely rely on the synthesis of lanthionine residues which can be incorporated by SPPS into linear peptides and enable cyclisation to give the characteristic lanthionine rings (*Scheme 1-6*). Several different approaches for the synthesis of lanthionine, methyllanthionine and orthogonally protected analogues have been investigated by many different groups and a select few are highlighted below. For a comprehensive review of this methodology see the review by Tabor.[127]



Scheme 1-6: Incorporation of bis-amino acids into peptides by SPPS
Reagents and conditions: (i) standard Fmoc SPPS protocol to build up chain (Fmoc deprotection using 40% piperidine, followed by amino acid addition using HBTU and DIPEA) followed by incorporation of lanthionine and further chain extension (ii) deprotection of both lanthionine protecting groups and Fmoc group (iii) cyclisation to form thioether bridge (iv) further chain extension or cleavage from resin

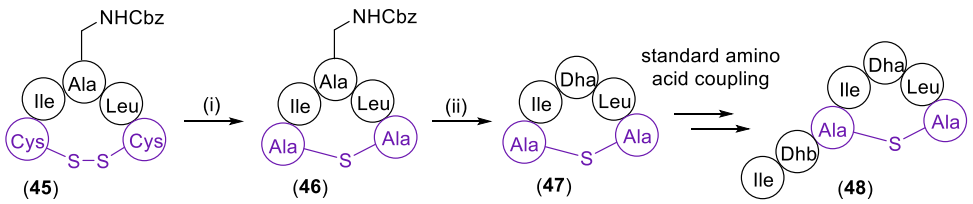
1.3.1 Desulfurisation to Synthesise Lanthionine and Lantibiotics

One approach towards the synthesis of lanthionine, that has been attempted by a number of groups, utilised the desulfurisation of cystine derivatives.[128,129] This approach involved the use of aminophosphines to desulfurise various cystine derivatives to give lanthionine compounds in modest yields (*Scheme 1-7*). These methods were incompatible with base sensitive protecting groups and several other difficulties, including purification, meant this approach was not pursued.



Scheme 1-7: General method for synthesis of lanthionine by desulfurisation

However, the desulfurisation methodology of Harpp and Gleeson was more successful when used to install the lanthionine bridge in a preformed peptide. This approach enabled the first total synthesis of nisin.[130] Fukase and co-workers synthesised fragments of the peptide chain with Cys-Cys bridged rings and then used desulfurisation to reduce the disulphide bonds to the thioether bonds of nisin (*Scheme 1-8*). Dehydro residues were then installed and the resulting fragments were coupled together using solution phase synthesis to give the completed peptide (**48**).

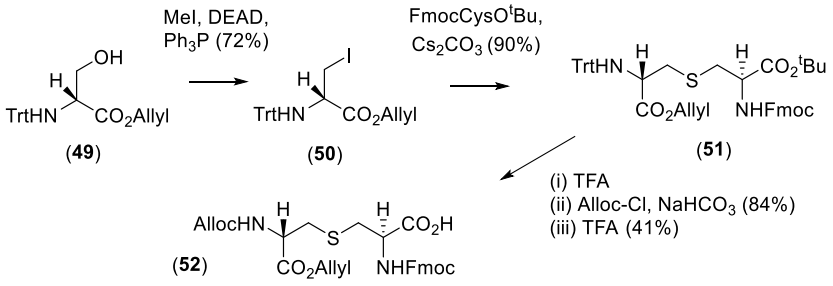


Scheme 1-8: Synthesis of nisin ring A
Reagents and conditions: (i) $P(NEt_2)_3$, DMF, (ii) 1. H_2, Pd, 2. HCHO, $NaBH_3CN$, 3. CH_3I, $KHCO_3$

1.3.2 Lanthionine Synthesis via β-Haloalanines

Several groups have investigated the synthesis of lanthionines *via* a coupling reaction between β-haloalanines and cysteine residues under basic conditions.[131,132,133] Problems were encountered with competing elimination reactions that gave dehydroalanine which could then undergo Michael addition with cysteine to give a mixture of diastereomers. To overcome this problem, derivatives with a trityl protected amine were developed where the trityl group sterically hinders α-proton abstraction.[134]

Tabor and co-workers showed that a trityl protected iodoalanine derivative (**50**) could undergo coupling with Fmoc-Cys-O^{t}Bu in the presence of Cs_2CO_3 to afford a protected lanthionine (**51**) in good yield (*Scheme 1-9*). Replacement of the trityl group with an alloc group and removal of the *tert*-butyl group gave orthogonally protected lanthionine (**52**) that could be readily incorporated in SPPS using the methodology described in *Scheme 1-6*.

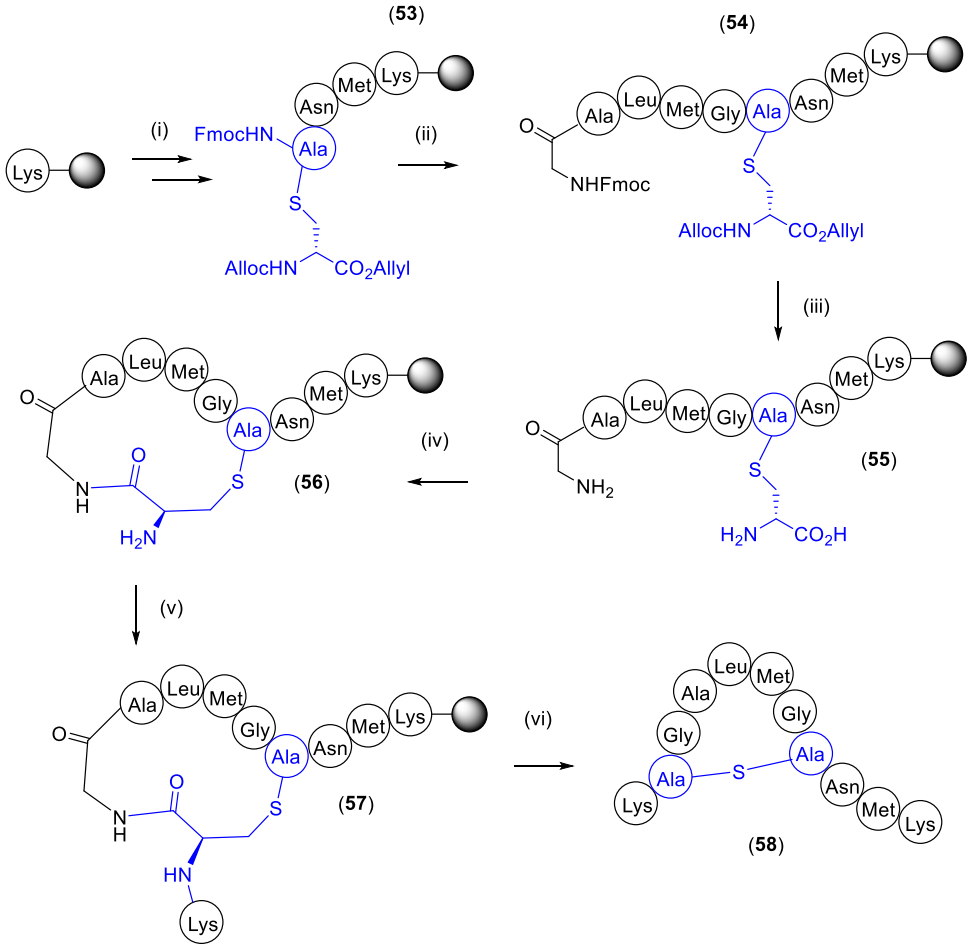


Scheme 1-9: Synthesis of orthogonally protected lanthionine

1.3.3 Incorporation of Lanthionine into Nisin Ring Analogues by SPPS

Tabor and co-workers went on to synthesise an analogue of ring C of nisin (**58**) using this lanthionine building block (*Scheme 1-10*).[133] Standard Fmoc SPPS methodology was used to

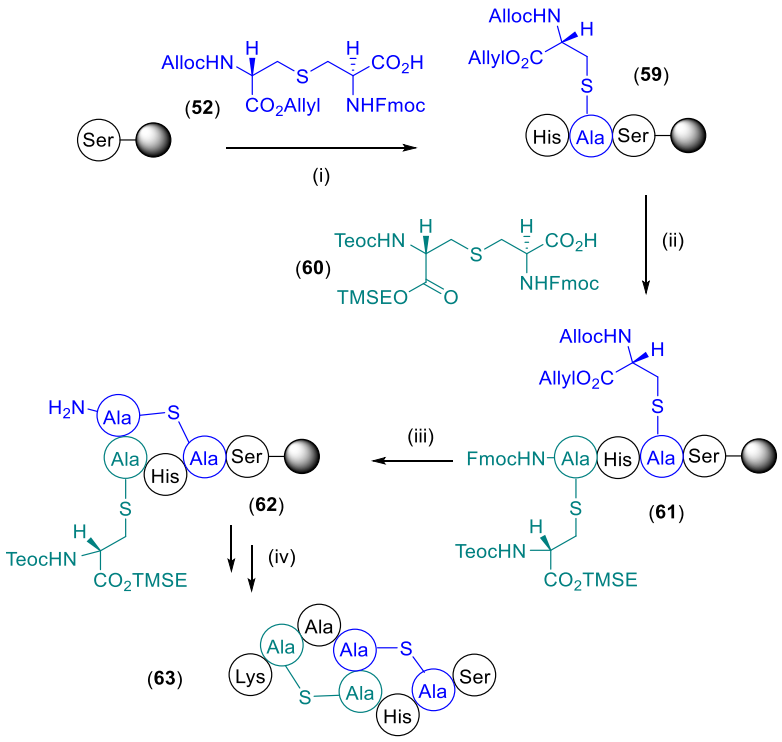
build up linear chain (**54**) and then the simultaneous allyl and Alloc deprotection of incorporated lanthionine (**52**) was carried out using a palladium catalyst to give (**55**). An intramolecular coupling could then be carried out to form the C ring.



Scheme 1-10: SPPS of an analogue of ring C nisin
*Reagents and conditions: (i) standard Fmoc SPPS protocol to build up chain followed by incorporation of lanthionine (**52**) with HOAt, PyAOP, DIPEA (ii) standard Fmoc SPPS protocol to build up chain (iii) 40% piperidine in DMF, then $Pd(PPh_3)_4$, 1,3-dimethylbarbituric acid (iv) HOAt, PyAOP, DIPEA (v) Fmoc-Lys-OH, HBTU, DIPEA (vi) 40% piperidine in DMF (vii) TFA, ethanedithiol, TIPS, water*

Using this methodology, Vederas and co-workers were able to form multiple lanthionine rings on resin to give the first SPPS of a lantibiotic, lactocin S.[135]

Taking this methodology, a step further, A. Tabor and co-workers synthesised the overlapping ring system of nisin rings D and E (**63**, *Scheme 1-11*). This required the synthesis of a second orthogonally protected lanthionine residue with silyl based protecting groups Teoc and TMSE (**60**).



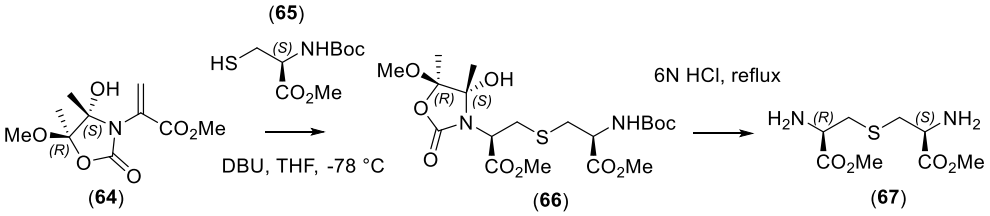
Scheme 1-11: SPPS of nisin rings D and E
*Reagents and conditions: (i) 1. incorporation of lanthionine (**52**) with HOAt, PyAOP, DIPEA, DMF 2. Fmoc-His(Trt)-OH, HBTU, DIPEA (ii) 1. incorporation of lanthionine (**60**) with HOAt, PyAOP, DIPEA (iii) 1. 40% piperidine in DMF 2. $Pd(PPh_3)_4$, 1,3-dimethylbarbituic acid, 3. HOAt, PyAOP, DIPEA (iv) 1. TBAF 2. HOAt, PyAOP, DIPEA 3. Fmoc-Lys(Boc)-OH, HBTU, DIPEA followed by Fmoc deprotection and cleavage from resin*

Linear peptide (**61**) was synthesised using standard Fmoc SPPS methodology. The allyl ester and Alloc protecting groups were then selectively removed and the peptide cyclized to give the ring E analogue (**62**). This was followed by chain extension and selective removal of the orthogonal Teoc and TMSE groups of incorporated (**60**), allowing a second on-resin cyclisation to take place to form ring D. Further chain extension and resin cleavage afforded (**63**).

1.3.4 Biomimetic Michael Additions

An early attempt at a biomimetic route to lanthionine used the Michael addition of cysteine residues to dehydroamino acids, but this proved unsuccessful with poor stereo control of the addition.[136] More recently, Avenoza and co-workers reported the first asymmetric Michael addition route to lanthionine and methyllanthionine (*Scheme 1-12*).[137] They used chiral dehydroamino acid derivatives (**64**) that acted like chiral auxiliaries providing stereocontrol of the addition. They were able to synthesise *meso*-lanthionine (*R,*

S) as well as the required (*S, R*) and (*S, S*) optically active isomers of methyllanthionine. The strongly acidic conditions for the removal of the chiral group means this route is not suitable for incorporating acid labile protecting groups.

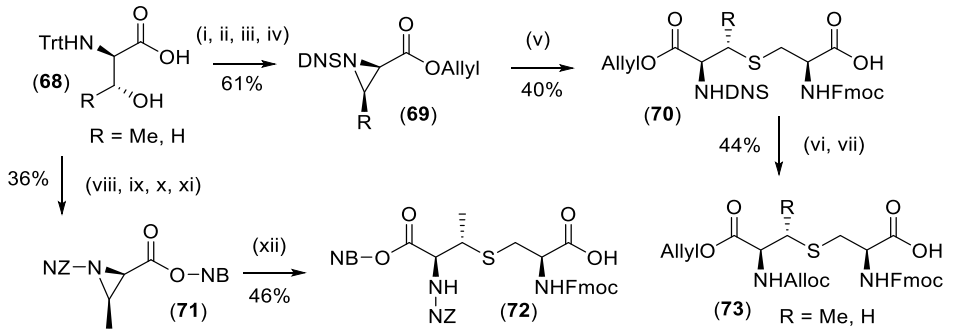


Scheme 1-12: Asymmetric Michael addition route to lanthionine

Biomimetic Michael additions have proven more successful when performed with a dehydro and a cysteine residue that are already installed in a linear precursor peptide. By using this strategy single rings of nisin,[138] subtilin[139,140] and an analogue of ring B of epidermin[141] have all been synthesised, notably, with the correct stereochemistry at the newly formed chiral centre. The excellent diastereoselectivity of these Michael additions suggests the linear sequence is pre-organised in a way that aids the regio and diastereoselectivity of these reactions. However, when cyclisation was attempted on more complex peptides, either with multiple dehydro residues,[140] when cyclising more than one ring[142] or with non-lantibiotic sequences,[143] the selectivity was lost suggesting a cyclase enzyme is still required for longer sequences.

1.3.5 Ring Opening Methods to Synthesise Lanthionine

The Vederas group successfully synthesised lanthionine and methyllanthionine derivatives using a synthetic route that relies on the ring opening of aziridine derivatives (**69**, **71**, *Scheme 1-13*).[144]



Scheme 1-13: Ring opening of aziridine derivatives
Reagents and conditions: (i) allyl chloride, $NaHCO_3$ (ii) MsCl, Et_3N, 0 °C (iii) 3:3:1 TFA/DCM/MeOH, 0 °C. (iv) DNs-Cl, Na_2CO_3 (v) Fmoc-Cys-OH, $BF_3.Et_2O$, 0 °C (vi) thioglycolic acid, DIPEA (vii) alloc chloride, DIPEA (viii) p-NBOH, p-TsOH (ix) TrtCl, Et_3N (x) MsCl, Et_3N, reflux (xi) 3:3:1 TFA/DCM/MeOH, 0 °C, p-NZCl, Na_2CO_3, (xii) Fmoc-Cys-OH, $BF_3.Et_2O$

Using this methodology, with a cysteine nucleophile, the Vederas group were able to synthesise the naturally occurring methyllanthionine diastereoisomer with both allyl/Alloc protecting groups and p-nitrobenzyloxycarbonyl (p-NZ)/p-nitrobenzyl (p-NB) protecting groups from two differentially protected aziridines both derived from D-Thr.[144]

1.3.6 Synthesis of Lacticin 3147

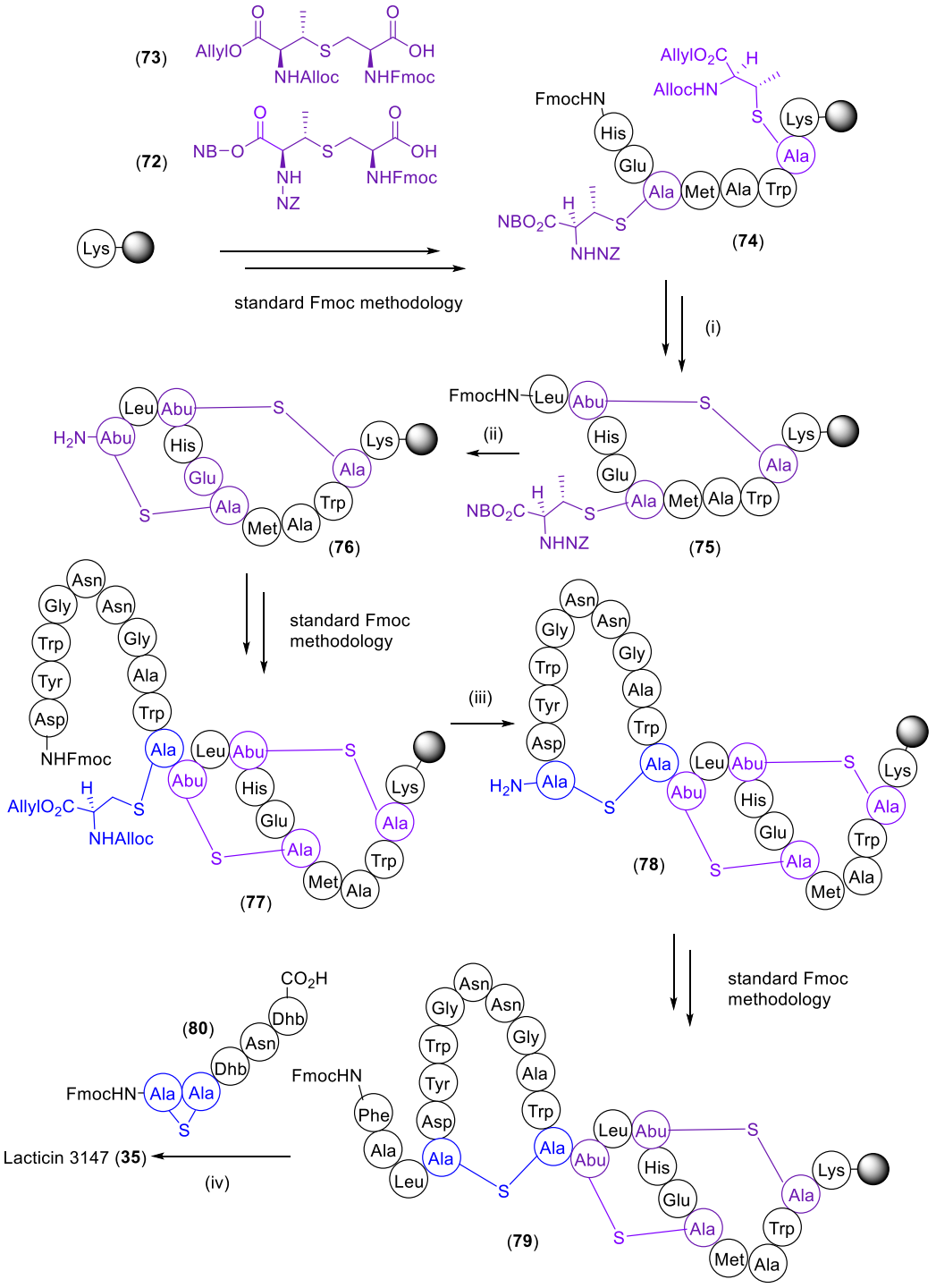
With the two methyllanthionine and lanthionine derivatives in hand, the group went on to synthesise both components of lacticin 3147 by SPPS. The synthesis of lacticin 3147 A1 (**35**) is described below (

Scheme 1-14).

Standard Fmoc methodology was used to synthesise linear peptide (**74**) with both methyllanthionine residues incorporated. The first ring was synthesised by deprotection of the allyl/Alloc protecting groups using $Pd(PPh_3)_4$ followed by Fmoc deprotection and cyclisation using PyBOP and HOBt. Fmoc-Leu-OH was then installed and the second ring was formed by removing both NZ and NB groups with 6M $SnCl_2$ in HCl and followed by cyclisation using the same conditions as before. Lanthionine (**52**) was then installed using standard Fmoc methodology and the chain was again extended to give (**77**). Repeating the cyclisation conditions for the first ring enabled the formation of the third and the chain was again extended by another three residues to give (**79**). The N-terminus pentapeptide (**80**) was pre-synthesised off-resin due to the complexity in incorporating dehydro residues and

a short thioether bridge. The two final fragments (**80**) and (**79**) were then coupled using fluoro-N,N,N',N'-tetramethyl formamidinium hexafluorophosphate (TFFH) and the final sequence was cleaved from the resin to give lacticin 3147 A1 (**35**).

The Vederas group also synthesised a ring-expanded, carbocyclic analogue of lacticin 3147 A2 (**36**), replacing the three thioether bridges with alkene bonds using on-resin ring-closing olefin metathesis.[145] The resulting carbocyclic peptide showed no biological activity. The oxa-analogue of this peptide has also been synthesised using protected oxa-lanthionines and although some biological activity was retained, there was a significant reduction.[146]



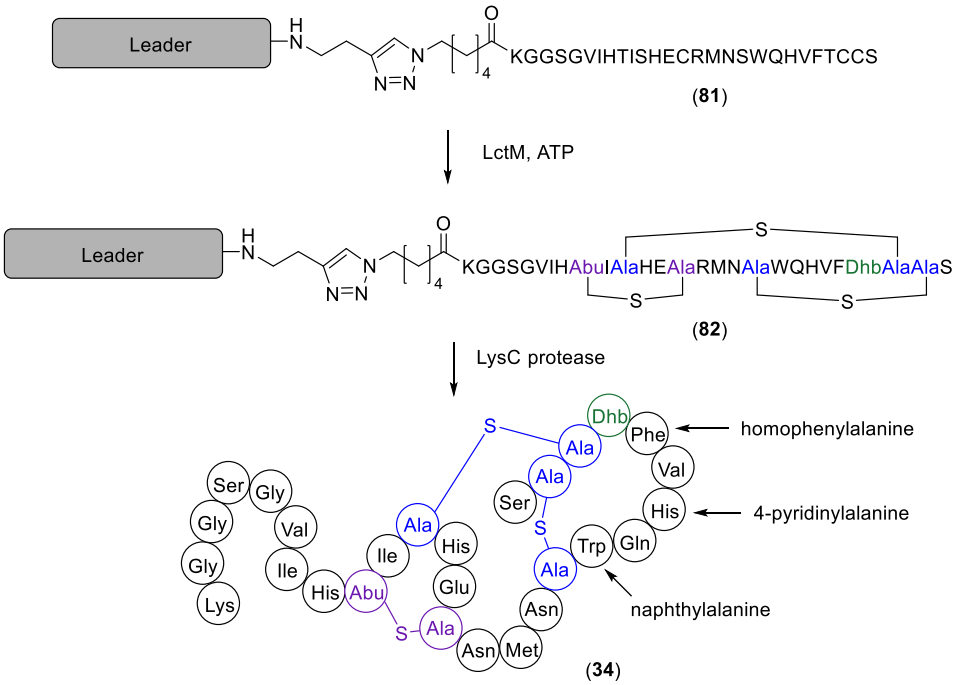
Scheme 1-14: SPPS of lacticin 3147 A1

Reagents and conditions: (i) 1. $Pd(PPh_3)_4$, $PhSiH_3$, 2. 20% piperidine 3. PyBOP, HOBt, NMM 4. PyBOP, HOBt, Fmoc-Leu-OH (ii) 1. $SnCl_2$, HCl 2. 20% piperidine 3. PyBOP, HOBt, NMM (iii) repeat of (i) 1-3 (iv) 1. 20% piperidine 2. TFFH, DIPEA, 3. TFA, water, anisole

1.3.7 *In vitro* Engineering

Biotransformations provide yet another tool for synthesising lantibiotics. This approach involves the isolation of the cyclase and dehydration enzymes from bacteria which can then be used on linear peptides *in vitro*. The advances in genome sequencing over the past decade have greatly improved the feasibility of this method, making it relatively easy to search genomes for the biological machinery we need such as conserved elements of dehydratase enzymes. This approach of 'genome mining' has also been successfully used to discover new lantibiotics such as Lichenicidin.[147] For a more in depth discussion of both *in vivo* and *in vitro* engineering see reviews by Ross and Vederas,[148] Field[149] and more recently Kuipers and co-workers.[150] These methods are also discussed in wider reviews by van der Donk and Paul[151] and Tabor.[127,152]

Van der Donk and co-workers were able to use this method to produce lacticin 481 and analogues by feeding in linear sequences such as (**81**) to an isolated LctM enzyme which carried out both dehydration of Ser and Thr residues and cyclisation reactions *(Scheme 1-15*).[153,154] Recognition of the non-natural sequences by LctM was dependant on recognition of the leader peptide which was attached by click chemistry. They were also able to feed in non-proteinogenic amino acids to generate slightly more potent analogues of lacticin 481 (**34**, *Scheme 1-15*).[155] Similar studies have been carried out with the modification enzymes of nisin with both the dehydration process[156] and the cyclisation process[157] being reconstituted *in vitro*. More recently van der Donk and Yang were able to introduce D-alanine into lantibiotics using a dehydroalanine reductase enzyme.[158]



*Scheme 1-15: In vitro mutasynthesis of lacticin 481 (**34**) and analogues indicated by arrows*

1.3.8 *In vivo* Engineering

The genetically encoded nature of lantibiotics means they are easily amenable to genetic engineering compared to other classes of antimicrobials. Mutagenesis studies *via* site directed or random mutagenesis can be used to generate analogues of wild type lantibiotics with improved physiochemical properties and improved activities. This approach has been fairly successful for nisin for which mutants with improved activity against both Gram negative bacteria[159] and Gram positive bacteria[160] have been generated. Some notable studies featuring hinge region mutants of nisin are highlighted below but for a recent review of nisin bioengineering see Field *et al*.[161]

There are several difficulties encountered with this approach which include mutations that impact the bacteria's biosynthetic pathway, where mutant intermediates are no longer recognised, decreasing production levels of lantibiotic. In some cases, mutants can be produced that are toxic towards bacteria expressing the lantibiotic.

The hinge region of nisin (residues 20-22; NMK) has been subjected to a significant number of mutation studies. Huan and co-workers used site directed mutagenesis to produce nisin Z mutants with slightly improved activity against Gram negative bacteria (left, *Figure 1-15*).[159] Using well-diffusion assays and peptide solutions at 20 μg mL^{-1} they showed the

mutants to have good activity against *S. dysenteriae*, *Salmonella*, *P. aeruginosa* and other Gram negative bacteria whilst wild type nisin Z showed no growth inhibition. Field and co-workers used saturation mutagenesis to generate a large bank of nisin A hinge mutants several of which were found to have increased activity against specific Gram positive pathogens.[160] Three of these are pictured in *Figure 1-15* (centre) and these were shown to have 100% increased activity, compared to nisin A, against *S. aureus* ST528 as assessed by well-diffusion assays.

A mutant, M21V (centre, *Figure 1-15)*, subsequently renamed, nisin V, showed increased activity against several significant pathogens and was taken on for further study. Nisin V was shown to be two-fold more potent, compared to nisin A, against several MRSA and VRE indicator strains as evaluation by broth micro-dilution assays.[162] The same group went on to carry out site-saturation mutagenesis of all three hinge residues to generate a further library of novel mutants.[163] Screening of this library indicated that small, chiral amino acids were preferential and building on this, two mutants, AAA and SAA, were rationally designed (right, *Figure 1-15*). These two mutants were shown to have improved activity, relative to nisin A, against all four strains of Gram positive bacteria used in agar diffusion assays but the effect was less pronounced in broth diffusion assays.

Zhou *et al*.[164] engineered an *L. lactis* strain to produce hinge region mutants of nisin to investigate how length of the hinge region affects activity. Shorter hinge region mutants showed greatly reduced activity against a range of Gram positive bacteria with their residual activity, in comparison to nisin A, dropped below 25%. Mutants with two extra amino acids were better tolerated with residual activity in the range of 25-50% compared to nisin A, apart from activity against *S. aureus* which dropped to 6%. Previous work in this group had shown that flexibility is important; mutants with proline-proline substitutions have greatly reduced activity[165] which correlates well with the understanding of the mode of action that requires the C-terminus to fold down into the membrane.

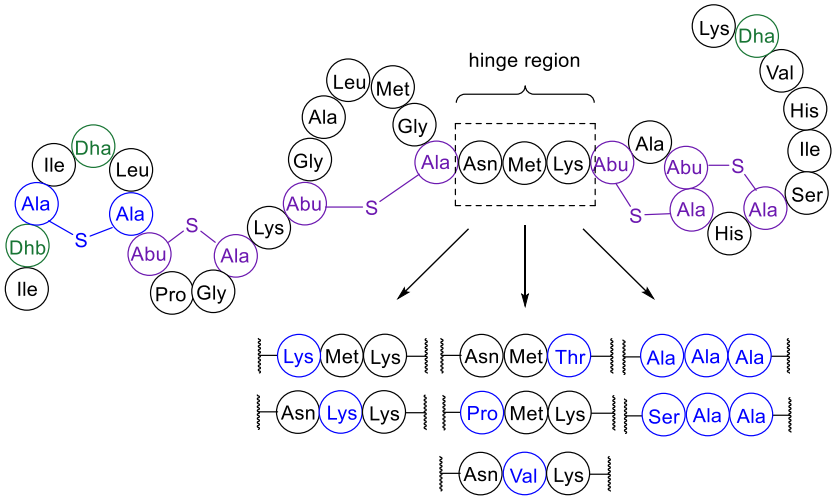


Figure 1-15: Hinge region mutants of nisin with improved activity; mutants with improved activity against Gram negative bacteria (left);[159] *mutants with improved activity against specific Gram positive strains (centre);*[160] *triple mutants showing improved activity against Gram positive bacteria in agar diffusion assays (right)*[163]

Using the same expression system in *L. lactis,* Zhou *et al.*,[166] have recently looked at improving the ability of nisin to cross the outer membrane of Gram negative bacteria and hence improve nisin's antibacterial activity against Gram negative bacteria. Ten different peptides known to be active against Gram negative bacteria, or sections of them, were fused onto the C-terminus of nisin or truncated versions (*Figure 1-16*). They were classed into 4 groups distinguished by their architecture: group 1 peptides consisted of Gram negative active peptides fused onto rings ABC, incorporating the hinge region of nisin; group 2 peptides consisted of Gram negative active peptides fused onto full length nisin; group 3 consisted of Gram negative active peptides fused onto full length nisin but with the last Dha residue left undehydrated as serine; group 4 consisted of peptides fused onto rings ABCDE of nisin with an additional serine-glycine linker. For group 3 peptides it was unclear whether the undehydrated Dha residue was intentional or as a result of a disrupted biosynthetic pathway. Many of the peptides synthesised were expressed poorly or degraded after expression which made obtaining meaningful amounts of the fused peptides challenging. Seven fused peptides were scaled up, purified and used in biological testing. The four most promising peptides (**83**-**86**) are pictured in *Figure 1-16*.

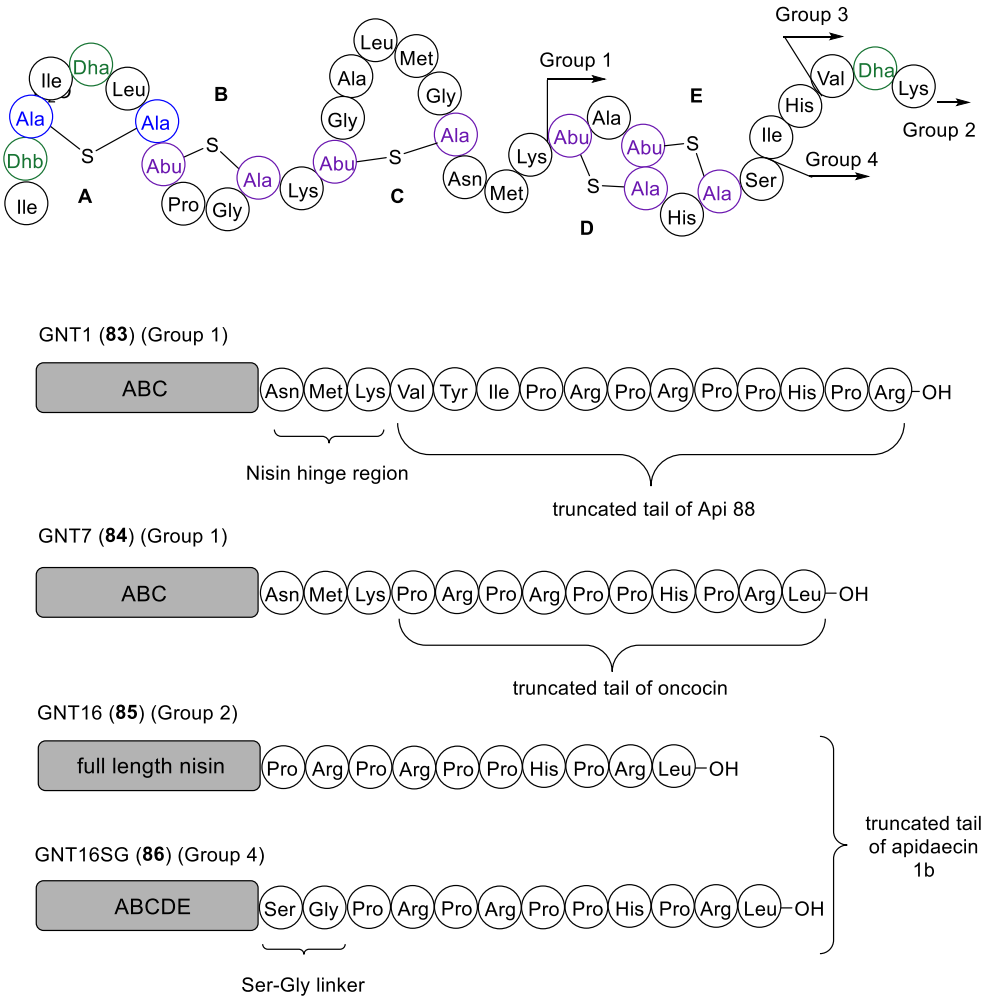


Figure 1-16: Nisin fragments fused with Gram negative active linear peptides

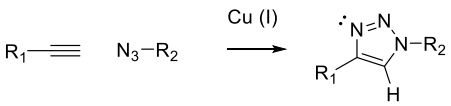
The best result obtained (GNT16, **85**) was from group two, with a truncated tail peptide of apidaecin 1b,[167] an anti-Gram negative peptide with a mode of action dependant on translocation into the cytoplasm, fused onto the C-terminus of full length nisin. The fused nisin was over twice as effective at killing *E. coli* but the modification had a detrimental effect on the activity against the Gram positive indicator strain used (*L. lactis*) indicating pore formation was probably reduced. This reduction could also have been occurring with the *E. coli* strain but was counteracted by improved ability to cross the outer membrane. GNT1 (**83**), GNT16SG (**86**), GNT7 (**85**) from groups 1 and group 4 did not have improved activity against *E. coli* but did retain some activity against *L. lactis.*

In addition to the naturally occurring analogues, the analogues produced from bioengineering provide us with good substrates for structure activity relationship studies though there is still much work to be done on deciphering how these structural changes impact the mechanism of nisin and other lantibiotics.

1.4 Modification of Peptides using Click Chemistry

The development of lantibiotics as clinically relevant compounds entails specific synthetic challenges but alongside this there are several wider barriers to the study and manipulation of peptides and proteins that all peptide chemists face. Peptides contain a variety of functional groups including amines, amides, alcohols, thiols and carboxylic acids. This means selectivity is a problem when modifying a peptide and with so many functional groups present several competing reactions can occur. A number of different tools have been developed in order to carry out regio- and chemoselective modifications of peptides both *in vitro* and *in vivo*[168] and one of the most significant and successful methods of these has been click chemistry.

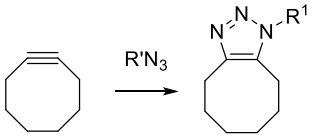
Click chemistry was first reported in 2002 by Sharpless[169] and by Meldal[170] as the use of copper (I) to catalyse a 1,3-dipolar-cycloaddition between an azide and an alkyne; a reaction that had previously required high temperatures and pressures.[171] Meldal also showed it was a reaction suitable to be performed on peptides still attached to solid supports with alkynes at the N-terminus. Click chemistry was later defined with a wider scope by Kolb, Finn and Sharpless as a reaction that "must be modular, wide in scope, give very high yields, generate only inoffensive by-products that can be removed by non-chromatographic methods, and be stereospecific (but not necessarily enantioselective). The required process characteristics include simple reaction conditions (ideally, the process should be insensitive to oxygen and water), readily available starting materials and reagents, the use of no solvent or a solvent that is benign (such as water) or easily removed, and simple product isolation."[172]



Scheme 1-16: Huisgen 1,3-dipolar-cycloaddition

Click chemistry has been widely employed throughout biomedical[173] and materials science[174] research and although click chemistry is now used as a broader term, the Huisgen 1,3-dipolar-cycloaddition is still considered to be the defining reaction. Modification of the reaction has included the use of different catalysts, copper free conditions and a variety of different substrates. In peptide chemistry, specifically, it has

been used for protein modification, labelling peptides and proteins, identifying enzyme inhibitors, drug discovery and molecular probes. Bio-orthogonal, copper free click reactions are now enabling selective click reactions to be carried out within living systems.[175] These tend to rely on strain-promoted alkyne-azide cycloadditions (*Scheme 1-17*). Nikic *et al.*[176] developed a procedure using click chemistry that enabled the labelling of cell-surface proteins in living mammalian cells. They used genetic code expansion technology[177] to incorporate ring-strained alkynes or alkenes into the sequences of the protein of interest and then carried out click reactions at physiological conditions with dyes containing azide or tetrazine groups respectively.



Scheme 1-17: Copper free click reaction with a strained cyclooctyne

The success of click chemistry in peptide and protein modification can be attributed to mild reaction conditions and the inertness of the resulting triazole moiety which mimics an amide bond well (*Figure 1-17*) and increases stability and resistance to proteases. In 2013 Valverde *et al*.[178] showed that the systematic replacement of residues with triazoles or "triazole scanning" can be an effective way to identify novel therapeutic peptidomimetics with improved stability.

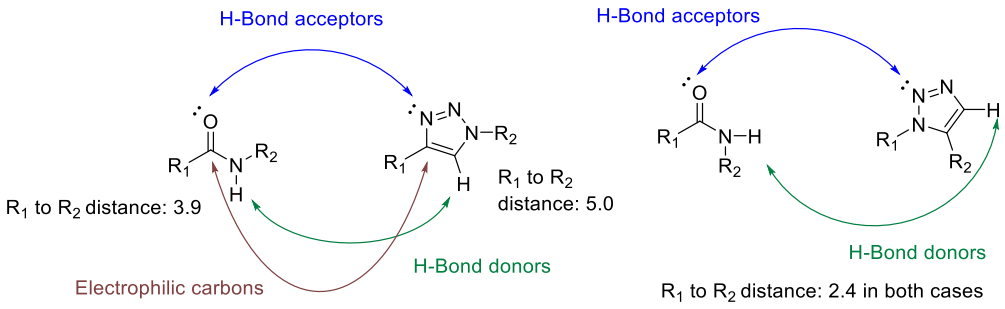
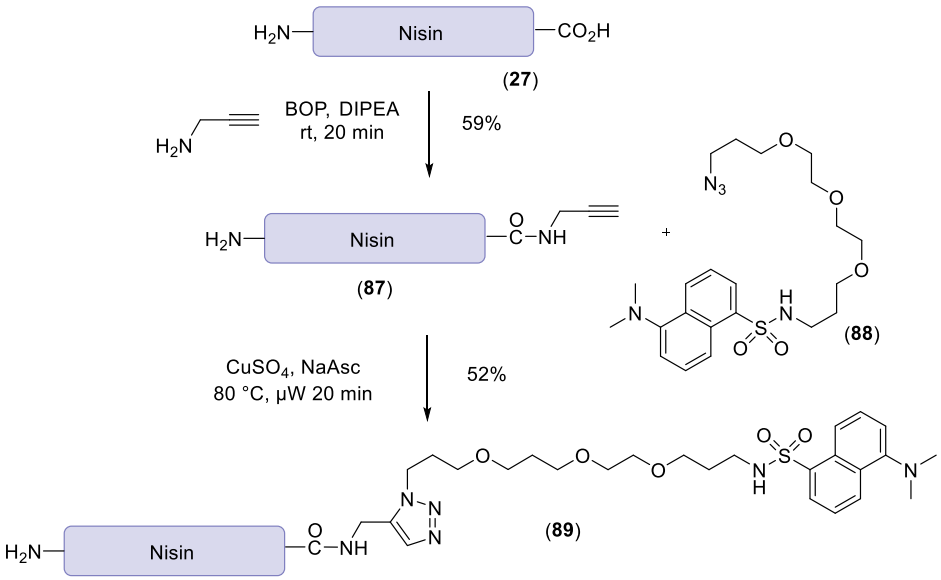


Figure 1-17: Cis amide bond (left) mimicked by a 1,4-disubstituted triazole and a trans amide bond (right) mimicked by a 1,5-disubstituted triazole

1.4.1 Click Chemistry for Manipulating Lantibiotics

Slootweg *et al*.[179] developed a useful method for functionalising WT nisin via click chemistry and were able to conjugate two different fluorescent reporter molecules, carboxyfluorescein and dansyl to the C-terminus of full length WT nisin with short PEG spacers (*Scheme 1-18*). Nisin contains only one carboxylic acid at the C-terminus and so regioselective modification is relatively straightforward; previous groups have also carried out fluorescent labelling at the C-terminus. However, amide coupling is not ideal; an excess of amine is required to prevent self-condensation with the side chain of one of nisin's three lysine residues and when the desired amine is a fluorescent molecule or other biologically relevant compound this can be expensive. An excess of amine can also make purification more difficult. Slootweg and co-workers reasoned that an inexpensive amine with a terminal alkyne could be used in excess to install a more reactive bio-orthogonal 'handle'. This allows for stoichiometric coupling with a large number of functionalised azides in this case, fluorescent reporter molecules.

Commercial sources of nisin typically contain only 2.5% nisin, 22.5% dairy proteins and 75% NaCl and so it was first necessary for Slootweg and co-workers to purify WT nisin using a previously published protocol of theirs.[180] With purified full length WT nisin in hand they coupled an inexpensive alkyne handle, propargylamine, onto the C terminus to give (**87**) and then carried out a click reaction with a dansyl fluorescent label modified with a short PEG linker and an azide (**88**). Using this method Slootweg and co-workers were also able to prepare a nisin dimer using a bis-azide with a short PEG linker. The resulting conjugates retained reasonable antimicrobial and pore forming activity.



Scheme 1-18: Using click chemistry to attach a fluorescent label to nisin

With the aim of producing more stable ring systems of lantibiotics, Slootweg *et al*.[181] have also investigated dicarba analogues of nisin and have shown that replacing lanthionine with dicarba bridge in ring AB analogues has minimal impact on binding to lipid II. Prior to this they also synthesised dicarba analogues of the overlapping ring system DE where alkene bridges replaced lanthionine (**77**, *Scheme 1-19*).[182] These analogues of the C-terminus did not show any biological activity on their own but Slootweg and co-workers also conjugated the DE C-terminus mimic to a lipid II binding motif to see if this would facilitate pore forming or antibacterial activity (*Scheme 1-19*).[183] Rings ABC of nisin were first obtained from a chymotrypsin digest of a commercial preparation of WT nisin (*Figure 1-18*) and using the same conditions as before they coupled propargylamine on to the C-terminus of the fragment to give (**90**).

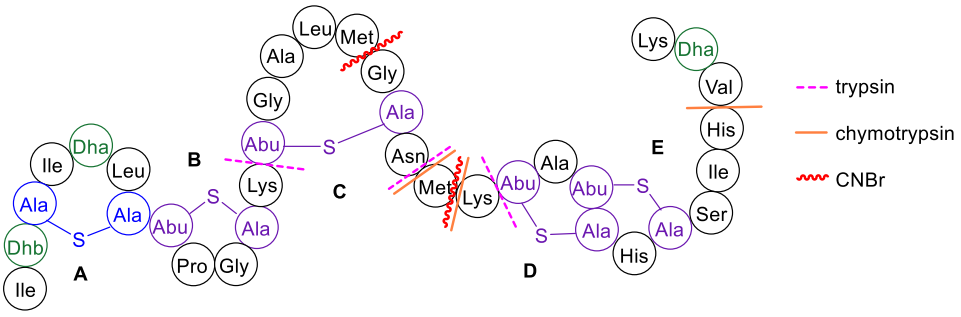
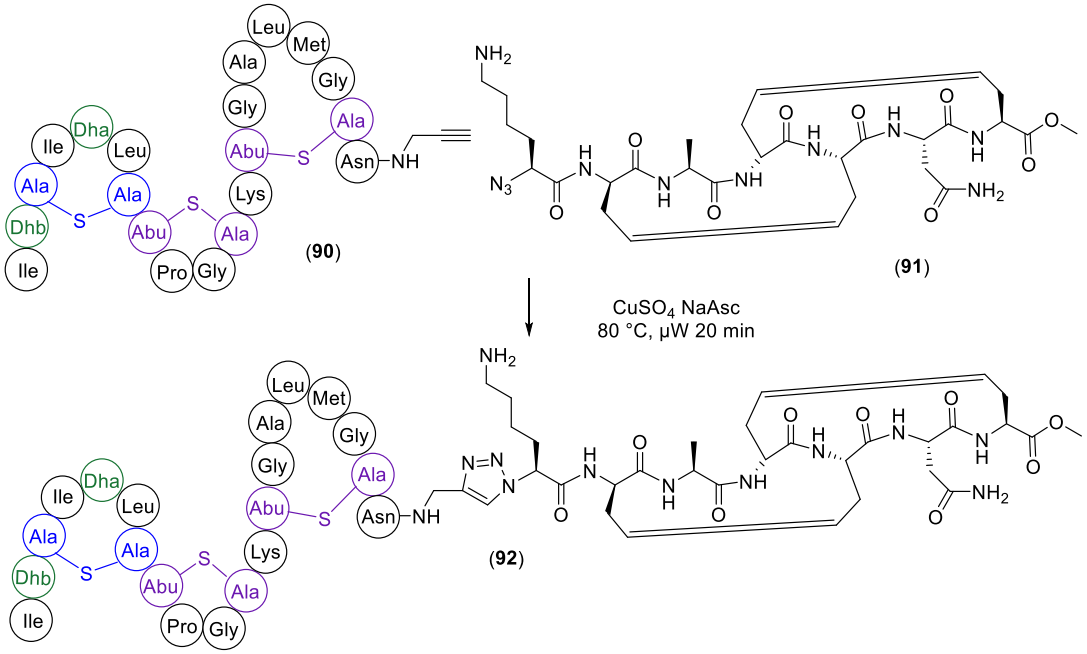


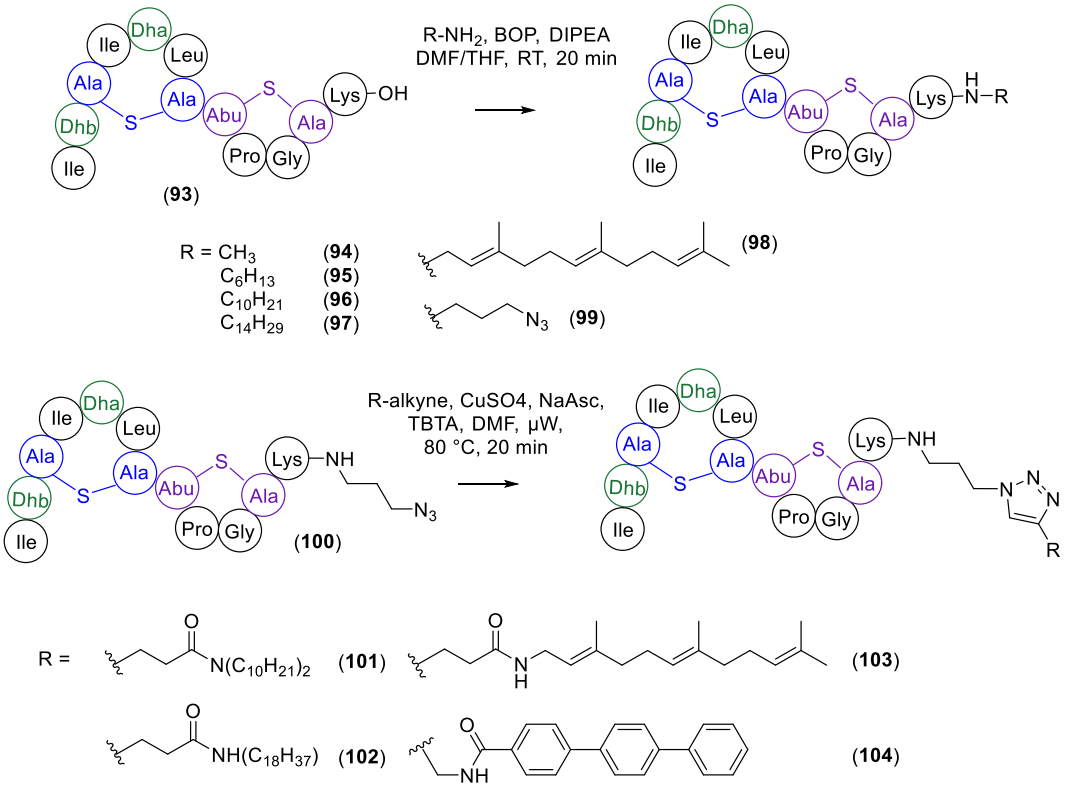
Figure 1-18: Cleavage sites of nisin for chymotrypsin (orange), trypsin (pink), and CNBr (red) elucidated by Slootweg et al.[180]

Click chemistry was again used to conjugate (**90**) the previously synthesised DE carba analogue (**91**). The conjugate did not show pore forming activity in experiments with large unilamellar vesicles (LUVs) encapsulating carboxyfluorescein (CF) but it did retain lipid II binding capabilities and performed similarly to nisin 1-20 in antimicrobial assays. This suggested the modified C-terminus, whilst not being biologically active itself, did not disrupt the activity of the nisin fragment.



Scheme 1-19: Click chemistry used to synthesise nisin hybrids with dicarba analogue tails

Koopmans *et al.*[98] have also looked at the production of more stable nisin analogues by replacing the entire C-terminus and hinge region with lipid tails (*Scheme 1-20*). By scaling up the enrichment and trypsin digest protocol as previously published by Slootweg, they obtained good amounts of WT AB rings of nisin (**27**). They then used a huge excess of amine lipids and otherwise standard amine coupling conditions to attach the smaller lipid amines directly onto the C-terminal lysine. If less than 50 equivalents were used, they found that the side chain amine of lysine would couple with the lysine carboxylic acid to give an unwanted cyclised side product. With larger, bulkier lipids they encountered solubility/miscibility problems and were unable to use such a large excess of reagents. They circumvented this problem by coupling a small azide "handle" to AB rings (**100**) and then used click chemistry to enable stoichiometric equivalents of reagents.



Scheme 1-20: Lipid analogues of nisin

Compounds (**96**-**98**) and (**104**) were found to have antibacterial activity comparable to that of nisin against clinically relevant, VRE and MRSA strains of bacteria *(Table 1-5*) and in serum stability assays were found to remain intact at a much higher rate than nisin. The analogues did not cause fluorescence leakage in lipid II spiked LUV assays suggesting they do not form pores like nisin but it was shown that lipid II binding was conserved as they antagonised pore formation by nisin in competition assays. Haemolysis assays suggested there was a small increase in lytic activity towards human blood cells compared to nisin.

	MIC (μg/mL)		
Compound	**MRSA USA300**	**MRSA WKZ2**	**VRE E155**
96	16	16	8
97	64	32	4
98	16	16	8
104	8	8	8
nisin	10	10	5

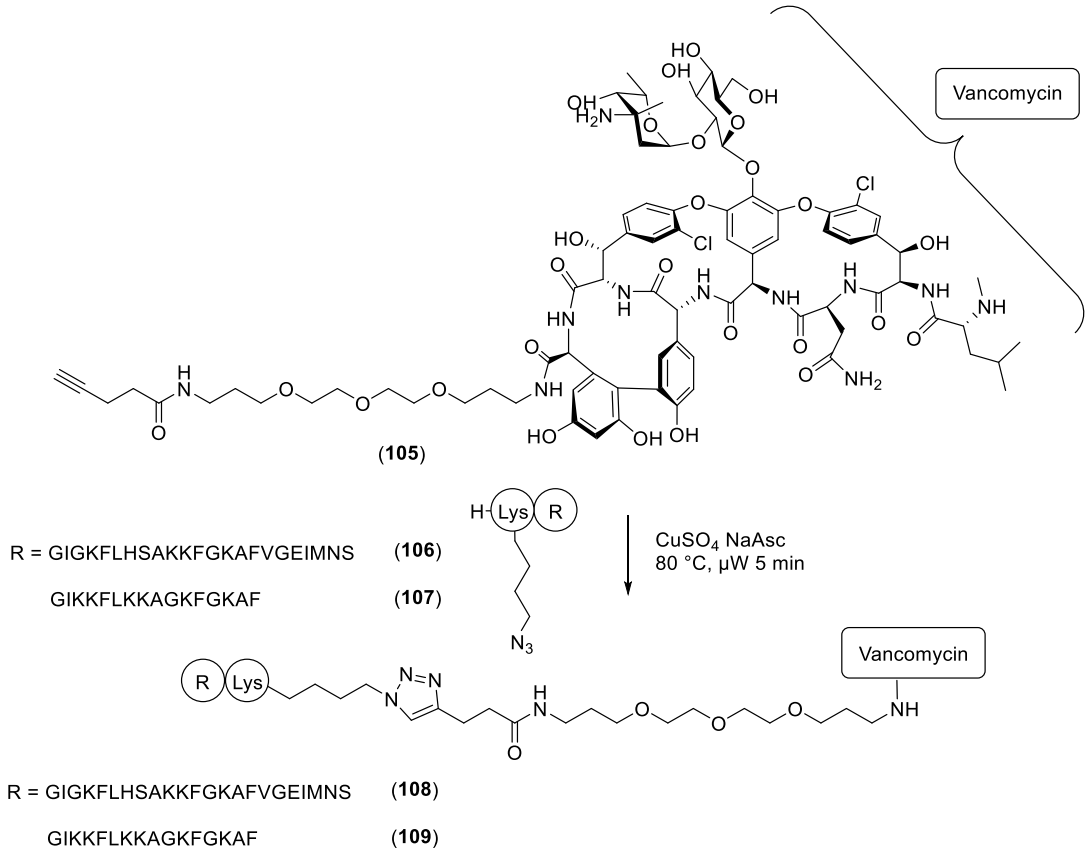
Table 1-5: Antibacterial activity of nisin analogues obtained by Koopmans et al.[98]

One considerable drawback of incorporating large alkyl groups is that aqueous solubility of these hybrids is greatly reduced. This could cause significant problems for these

compounds if used in a clinical setting; poor aqueous solubility is often associated with slow drug absorption and inadequate bioavailability. It will be interesting to see how these compounds are further optimised and their mode of action deciphered.

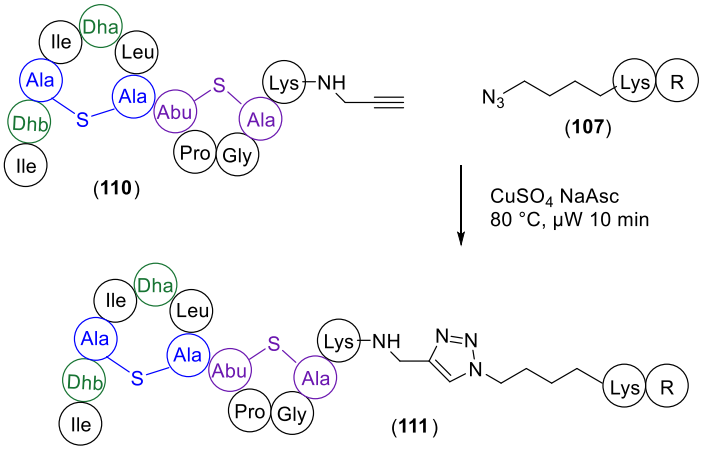
Breukink and co-workers used the lipid II binding action of nisin 1-12 conjugates to revive activity of vancomycin against VRE.[184] Vancomycin resistance has been shown to be conferred through the substitution D-Lac for the terminal D-Ala residue in lipid II resulting in a decreased level of vancomycin binding.[185] It was thought that this could be compensated for by conjugating another lipid II binding moiety, simultaneously binding two separate sites of the target to increase overall binding affinity. Computer modelling was used to predict the optimal binding points and connectivity lengths between nisin 1-12 and vancomycin. Click chemistry was used to produce a set of three conjugates. In MIC assays the best conjugate was shown to be about 40 times more active than either of its components.

Breukink went on to use the most successful vancomycin-alkyne adduct (**105**, *Scheme 1-21*) in a different study to investigate whether conjugating vancomycin to a linear, cationic AMP could increase the selectivity and potency.[186] As discussed previously, linear AMPs disrupt bacterial membranes and form pores similar to nisin but *via* different mechanisms, notably, without binding lipid II. This tends to result in lower potency and less selectivity which both contribute towards toxicity for mammalian cells. Vancomycin adduct (**105**) was conjugated using click chemistry with magainin 2 modified with an N-terminus lysine bearing an azide side chain (**106**). A previously optimised analogue of magainin 2 was also used (**107**).



Scheme 1-21: Using vancomycin to improve selectivity and potency of magainin

The conjugates (**108**, **109**) showed improved activity compared to that of magainin 2 and its analogue alone but did not perform better than vancomycin against Gram positive bacteria (*Table 1-6*). However, against VRE the conjugates did perform better than vancomycin. Additionally, the group also synthesised a nisin 1-12 conjugate with the optimised magainin 2 analogue (**111**, *Scheme 1-22*) which did not perform as well as the best vancomycin conjugate (**109**) and this was attributed to the weaker binding of nisin 1-12 to lipid II compared to vancomycin, though this was not investigated further.



Scheme 1-22: Nisin 1-12 conjugated with the optimised magainin 2 analogue

	MIC (µg/mL)		
Compound	**MRSA (15A763)**	**VRE (15A799)**	**VSE[a] (15A797)**
vancomycin	0.4	128	0.5
106	>256	256	256
107	256	64	128
108	32	256	16
109	2	16	4
111	-	32	64

Table 1-6: Antibacterial activity of vancomycin and nisin conjugates obtained by Breukink[186]
[a]VSE: vancomycin susceptible Enterococci

1.5 Aims and Objectives

AMPs have significant potential as therapeutics but are held back from clinical application by poor pharmacokinetics, poor drug-like properties and an incomplete understanding of their mechanisms of action and selectivity. Various strategies have been employed to improve their drug like properties with varying success. Lantibiotics have the additional synthetic challenge of their thioether bridges which has been tackled using several synthetic and biosynthetic approaches.

The aim of this project was to prepare simplified mimics of nisin with improved solubility and stability with an accessible synthetic route. The design of simplified analogues centred around the removal or replacement of synthetically challenging lanthionine residues and unstable dehydro residues. To preserve the selectivity and activity of nisin it was necessary

to design mimics with conserved AB ring moieties. The C-terminus, however, was replaced with simpler, linear pore forming peptides which were designed with a non-peptide hinge region mimic attached to rings AB *via* click chemistry (*Figure 1-19*).

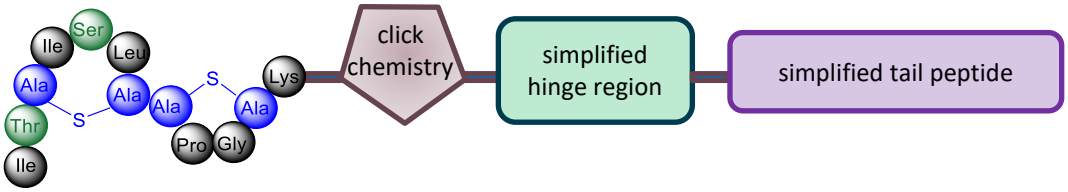


Figure 1-19: Design of simplified analogues of nisin

It was proposed that rings AB could be obtained from a digestion of WT commercially available nisin as reported by Slootweg *et al*.[180] and, in addition, this moiety could also be simplified further by using SPPS methodology developed with the Tabor group.[133,187,188] Using this methodology, *via* the synthesis of an orthogonally protected lanthionine residue, the methyllanthionine of WT nisin could be replaced with lanthionine and the unstable dehydro residues could be replaced with more stable analogues such as their biosynthetic precursors, serine and threonine. Analogues of rings AB produced this way will be valuable in their contribution towards hybrid lantibiotics but also in studies examining the molecular basis of lipid II binding.

Modifications to the structure of nisin may have a negative impact on biological activity, however, this could be balanced by the improvement in pharmacokinetic properties of the hybrid analogues.

2 Lanthionine Synthesis

This chapter describes the synthesis of orthogonally protected, target lanthionine (**60**, *Figure 2-1*) that will be incorporated into analogues of rings AB of nisin *via* SPPS using the procedure as outlined in Chapter 1.3, *Scheme 1-6*. This analogue had previously been synthesised within the Tabor group by Mothia to enable the synthesis of an analogue of the overlapping ring system of nisin rings DE *(Scheme 1-11*).[189] The *S,R* diastereomer was synthesised in accordance with WT nisin which contains only *S,R* methyllanthionine and lanthionine. Alongside the transient Fmoc protecting group, another set of protecting groups is required to enable the on-resin cyclisation that forms the characteristic thioether bridge. In this case, silyl-based protecting groups were chosen for the protection of the amino and carboxylic acid moieties, (trimethylsilyl)ethoxycarbonyl (Teoc) and trimethylsilylethyl (TMSE) respectively, as they could be easily removed under mild conditions and at a neutral pH using a source of fluoride ions such as TBAF (*Figure 2-1*).

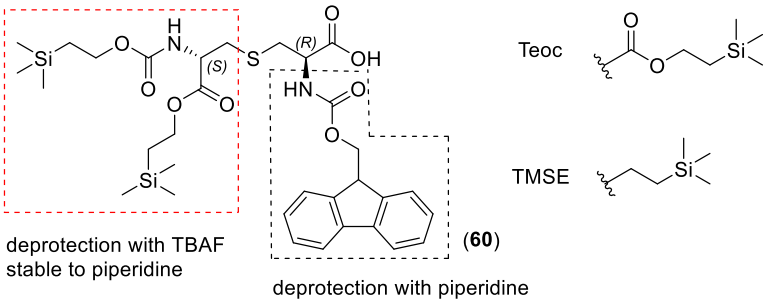
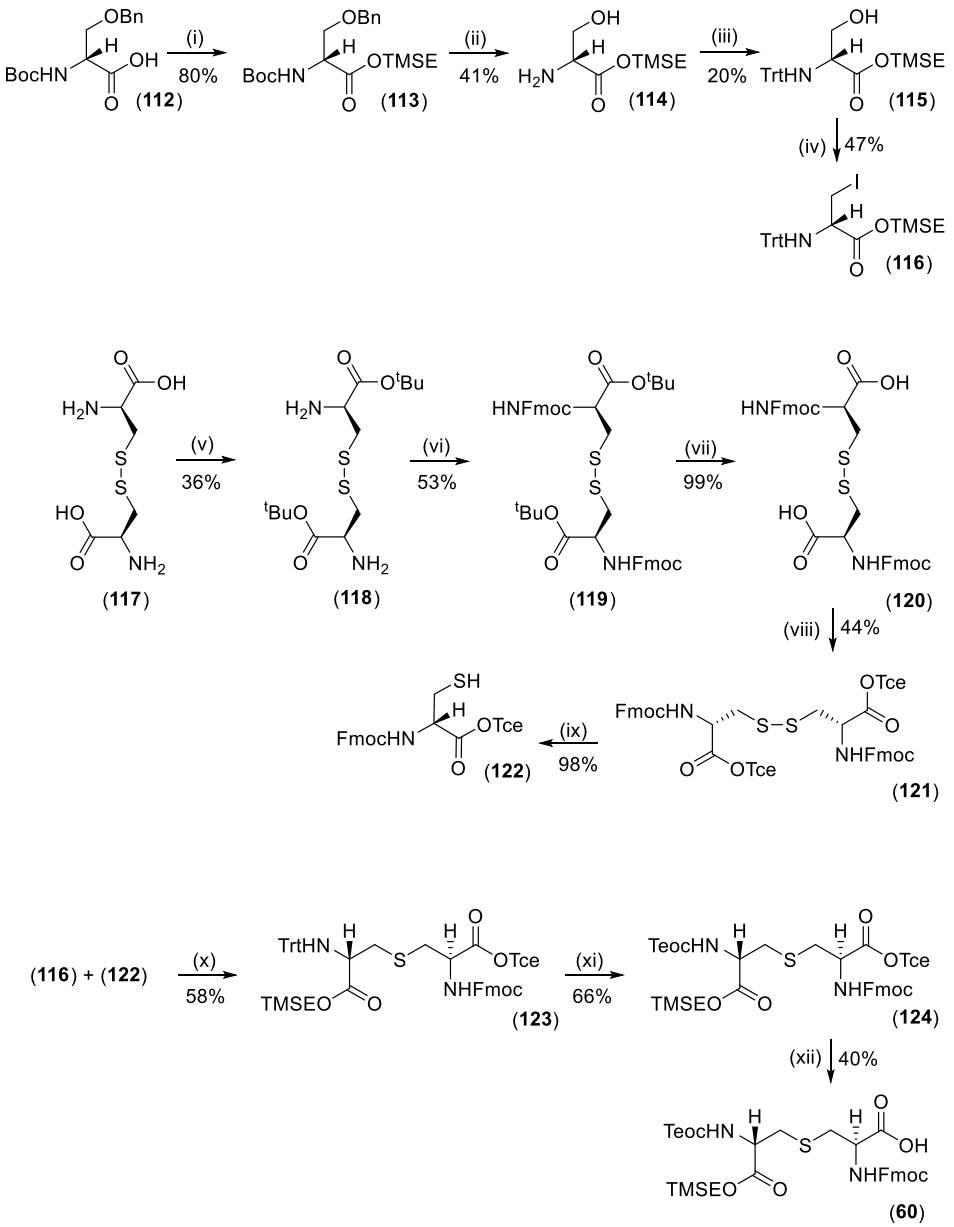


Figure 2-1: Orthogonally protected (Teoc, TMSE/Fmoc) lanthionine

2.1 Published Procedure

The route designed by Mothia and Tabor used Boc-D-Ser(Bn)-OH (**112**) and L-cystine (**117**) as starting materials (*Scheme 2-1*). This had been previously carried out on a small scale to give an overall yield of <1% from Boc-D-Ser(Bn) (**112**) and 1% from L-cystine (**117**).[188]

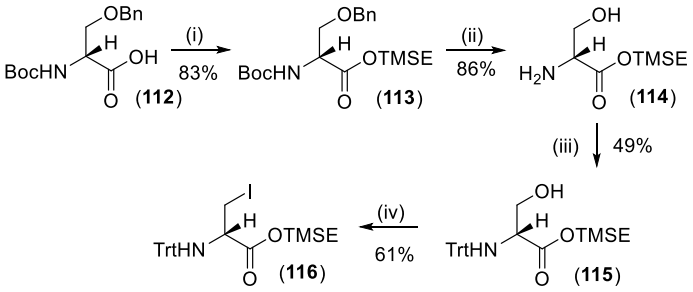


*Scheme 2-1: Synthesis of (Teoc, TMSE/Fmoc) lanthionine (**60**); route and yields by Mothia*[189]
Reagents and conditions: (i) 2-(trimethylsilyl)ethanol, DMAP, DCC (ii) 1. Pd/C 2. TFA (50%) (iii) Ph_3MeCl, NEt_3 (iv) PPh_3, DEAD, MeI, -2 °C (v) $HClO_4$, t-butyl acetate (vi) FmocCl, NMM (vii) 50% TFA, 50% CH_2Cl_2 (viii) p-TSA, TceOH, 100 °C, (ix) βME, PPh_3, H_2O (x) Cs_2CO_3, 0 °C (xi) 1. 10% TFA, 5% TES, 2. $NaHCO_3$, Teoc-ONSu, (xii) NH_4OAc, Zn dust

This route was initially repeated on a small scale (2 g) and was later adapted to suit a larger scale (25 g). Reagents and purification methods were modified to ease the difficulties associated with handling large scale reactions (Chapters 2.1.1-2.1.3). To optimise the synthesis further, a second route was designed (Chapter 2.2)

2.1.1 Synthesis of β-Iodoalanine (116)

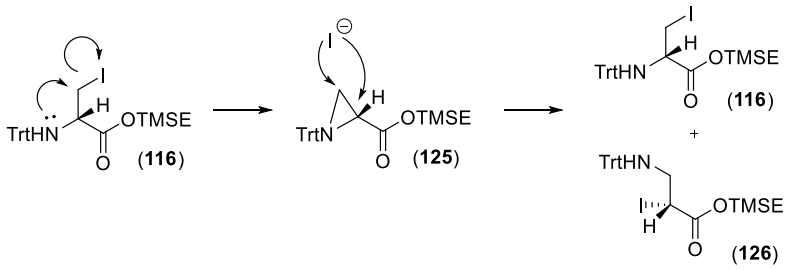
Synthesis of protected β-Iodoalanine (**116**) was first attempted using the Mothia and Tabor route. This began with the esterification of commercially available Boc-D-Ser(Bn) (**112**) with 2-(trimethylsilyl)ethanol (TMSEOH) using DMAP and DCC to give Boc-D-Ser(Bn)-OTMSE (**113**) in good yield (83%). This was followed by a two-step deprotection procedure. The benzyl group was removed by hydrogenation using a palladium on carbon catalyst (10% loading) and the crude product was then treated with TFA, removing the Boc group, to give H-D-Ser-OTMSE (**114**) in good yield (86%). Tritylation of the amine was then carried out using basic conditions and trityl chloride. As previously mentioned, the trityl group is an important transient protecting group, conserving the stereochemistry at the alpha carbon. Trt-D-Ser-OTMSE (**115**) was then converted to protected β-iodoalanine (**116**) using a Mitsunobu type reaction.



*Scheme 2-2: Synthesis of protected β-Iodoalanine (**116**)*
Regaents and conditions: (i) 2-(trimethylsilyl)ethanol, DMAP, DCC (ii) 1. Pd/C 2. TFA (50%) (iii) Ph_3MeCl, NEt_3 (iv) PPh_3, DEAD, MeI, -5 °C

Low yields of around 20-30% were initially obtained in the Mitsunobu reaction which was partly attributed to the reaction not going to completion. Analysis by TLC showed starting material (**115**) was still present in the reaction mixture after 6 h. Longer reaction times were not feasible as the product needed to be synthesised and purified in the same day to avoid decomposition of the product and so the reaction was instead carried out at a higher concentration using a smaller volume of solvent. Changing the initial concentration of Trt-D-Ser-OTMSE (**115**) from 0.47 M to 0.94 M was shown to push the reaction to completion as visible by TLC analysis. Formation of aziridine (**125**) was also identified as a reason for low yields and it was believed this was being formed both during the reaction and during purification (*Scheme 2-3*). This unwanted side product had previously been identified within the group it had been shown that the aziridine can be attacked by iodide giving two regioisomers (**116** and **126**).[133,187] The presence of aziridine (**125**) was easy to

identify as it gave a bright pink colour to the solution (presumably attributed to an iodo- species not the aziridine) and the structure was confirmed by NMR analysis. To minimise this problem, the reaction mixture was kept below -2 °C throughout the course of the reaction using a salt and ice bath and care was taken to carry out the purification process as quickly as possible using reduced pressure at room temperature to concentrate the column fractions *in vacuo*. By using more concentrated reaction conditions and carrying out the purification as quickly as possible, yields were improved from 20-30% to around 60%.



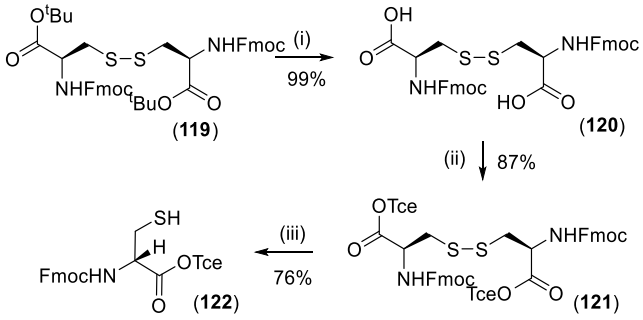
Scheme 2-3: Aziridine formation and possible ring opening

When the scale of this reaction was increased (1 g to 10 g), care was taken to add DEAD and MeI slowly as monitoring the reaction temperature showed addition of these reagents increased the temperature inside the reaction vessel by 10 °C. This was important to avoid due to formation of the aziridine and due to safety concerns as DEAD is known to be explosive at high temperatures.

2.1.2 Synthesis of Protected Cysteine (122)

Concurrently, protected cysteine (**122**) was synthesised from a stock of (Fmoc-Cys-O^{t}Bu)$_2$ (**119**) (custom synthesis by Oxygen Healthcare, Cambridge, UK). A solution of 50% TFA in CH_2Cl_2 was used to remove the *tert*-butyl groups which was followed by esterification of the disulfide (**120**) with trichloroethanol and *p*-toluenesulfonic acid (*p*-TSA). This was carried out under reflux, overnight using a Dean Stark trap to remove the water produced by the reaction. To avoid using the carcinogenic solvent benzene, as previously used by Mothia, the reaction was attempted using toluene but this significantly decreased the yield (45% from around 60%) and so benzene was used for all further reactions. The first step of the work up procedure required cooling of the reaction mixture to 0 °C and filtration to remove the excess *p*-TSA. Purification required a lengthy flash column chromatography procedure

using two different solvent systems and the product was found to elute slowly over many column fractions. When the scale was increased to multigram amounts, this purification became impractical as it used many litres of mobile phase and required about 5-6 h. It was thought the difficulty was due to poor solubility of the product and so to take advantage of this, the product was instead added to an excess of MeOH and left in the fridge overnight. In the morning, the mixture could be filtered and washed with further portions of cold MeOH to give pure (**121**), as assessed by NMR, and column chromatography was avoided. This method of purification saved significant time and improved the yield to 87%. Care had to be taken to ensure all the acid had been removed otherwise the methyl ester was readily formed.



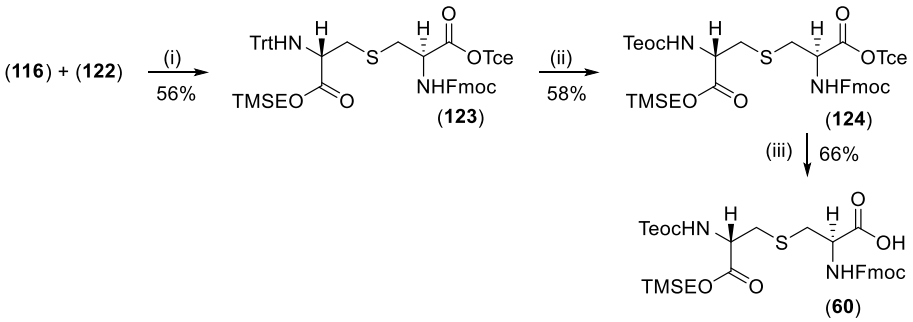
*Scheme 2-4: Synthesis of protected cysteine (**122**)*
Reagents and conditions: (i) 50% TFA, 50% CH_2Cl_2 (ii) p-TSA, TceOH, 100 °C, (iii) βME, PPh_3, H_2O

$(Fmoc\text{-}Cys\text{-}OTce)_2$ (**121**) was then reduced using 2-mercaptoethanol (βME) to the thiol (**122**). Again, when the scale of this reaction was increased, purification using column chromatography was unsuccessful and the product (**122**) co-eluted with impurities as analysed by TLC and NMR. The conditions of Wright were then employed which used dithiothreitol (DTT) as the reducing agent (*Scheme 2-5*).[190] Excess DTT, the oxidised by-product and excess base could be removed during the workup and column chromatography was again avoided. This procedure improved the yield from 76% to 92% and significantly decreased the time taken for this step.

*Scheme 2-5: Synthesis of protected thiol (**122**)*

2.1.3 Coupling of Cysteine (122) and β-Iodoalanine (116)

Coupling of the β-iodoalanine (**116**) and cysteine (**122**) was carried out in mild base at around 0 °C. Initially poor yields were obtained (30-40%) which were accounted for by the re-oxidation of thiol (**122**) to the unreactive disulphide (**121**), though this could be recovered during purification, and the decomposition of β-iodoalanine (**116**) to the aziridine as identified by a visible pink colour in the solution. Previously it has been shown that the ring opened regioisomer (**126**) of iodoalanine (**116**) can react with cysteine and form an unwanted regioisomer of lanthionine, norlanthionine.[133,187] However, at low temperatures ring opening is believed not to occur and no nor-lanthionine was observed during NMR analysis of the impurities. The presence of water in basic conditions was also enough to remove the Fmoc group and the resulting product was also observed by LCMS in trace amounts. Yields were thus improved (to 56%) by lowering the temperature of the reaction to -5 °C to discourage aziridine formation and care was taken to ensure the reaction was kept as dry as possible with the use of molecular sieves. Caesium carbonate was also dried overnight, prior to use, in a desiccator under reduced pressure.

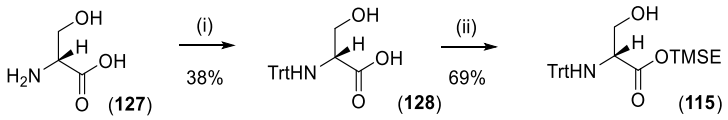


*Scheme 2-6: Coupling of the β-iodoalanine (**116**) and cysteine (**122**) followed by protection group manipulation to give protected lanthionine (**60**)*
Reagents and conditions: (i) Cs_2CO_3, -5 °C (ii) 1. 10% TFA, 5% TES, 2. $NaHCO_3$, Teoc-ONSu (iii) NH_4OAc, Zn dust

A two-step reaction was used to remove the trityl group of coupled intermediate (**123**) under mild acidic conditions, necessary to avoid removing the silyl groups. The acid was removed during a workup step with sodium bicarbonate. The crude residue was re-dissolved in 1,4-dioxane and the Teoc group was added using Teoc-ONSu to give (**124**). Small amounts of TMSEOH were found to have eluted with lanthionine (**124**) during purification as identified by NMR and this was removed *in vacuo*. Deprotection of the Tce group was carried out with zinc to give the orthogonally protected lanthionine (**60**) that was ready to be incorporated into a peptide.

2.2 Optimisation of Synthetic Route

It was then investigated if it was possible to synthesise the desired lanthionine analogue with cheaper starting materials and a fewer number of steps. The overall scheme for the adapted route can be seen in *Scheme 2-10*.



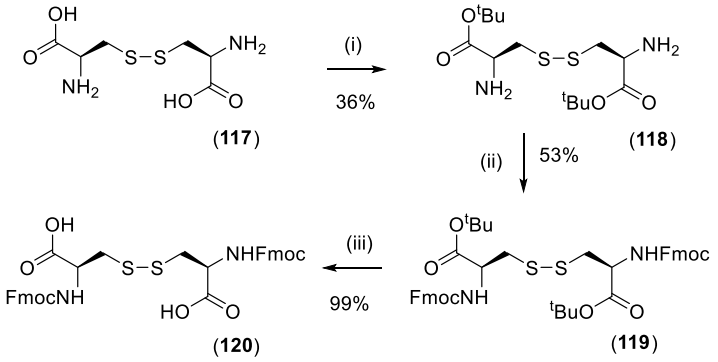
Scheme 2-7: Route to Trt-D-Ser-OTMSE using unprotected D-Serine as a starting material Reagents and conditions: (i) 1. TMSCl, NEt_3, 2. MeOH, 3. Ph_3MeCl, NEt_3 (ii) EDCI, DMAP, TMSEOH

Previously within the group there had been success using a "one-pot" procedure that used trimethylsilyl chloride to temporarily protect the alcohol and acid group of D-serine (**127**) ensuring tritylation only occurred at the amine.[191] However, it had also been noted that this was not a reliable reaction and Trt-D-Ser (**128**) was recovered with 38% yield using this method.

With Trt-D-Ser (**128**) in hand, the next step was to esterify the acid with TMSEOH. Amino acids can be esterified using standard Fischer conditions[192] (eg H_2SO_4, reflux) or converting first to the acid chloride with thionyl chloride. In this case, acidic conditions needed to be avoided due to the acid lability of the trityl group. Steglich conditions[193] (DCC, DMAP) could also be employed but without protection of the hydroxyl group a large excess of alcohol would be required to avoid self-condensation and polymerisation. Taking this into account, an excess of TMSEOH (>10 equivalents) was used to esterify Trt-D-Ser (**128**), forming the TMSE ester (**115**) in good yield (69%). Excess TMSEOH was collected during purification and reused.

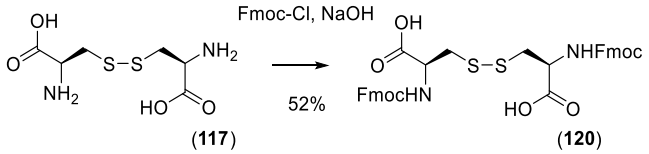
This route to Trt-D-Ser-OTMSE (**115**) had an overall yield of 26% over two steps which was lower than the original route by Mothia of 35% over three steps. However, this is balanced by a shorter, quicker, route and considerably cheaper starting material of D-serine; £28.70 for 5g from Sigma Aldrich compared to £150 for Boc-D-Ser(Bn). There is also potential to optimise the first tritylation of serine reaction.

Limited availability of Fmoc-Cys-O^{t}Bu (**119**) meant that the route to thiol (**122**) needed to be reassessed. Previously Fmoc-cystine (**120**) had been synthesised *via* *tert*-butyl protection of cystine (**117**) using perchloric acid and *tert*-butyl acetate (*Scheme 2-8*).



*Scheme 2-8: Previously used route to Fmoc-cystine (**106**)*[189]
Reagents and conditions: (i) $HClO_4$, tert-butyl acetate (ii) FmocCl, NMM (iii) 50% TFA, 50% CH_2Cl_2

Wright improved the yields of *tert*-butyl protection to 93% and used Fmoc succinimide to give a higher yield of 61% for Fmoc protection[190] but it remained undesirable to use perchloric acid on a medium to large scale given its toxicity and potential for explosion. A short investigation was carried out into whether L-cystine (**117**) could be directly Fmoc protected.

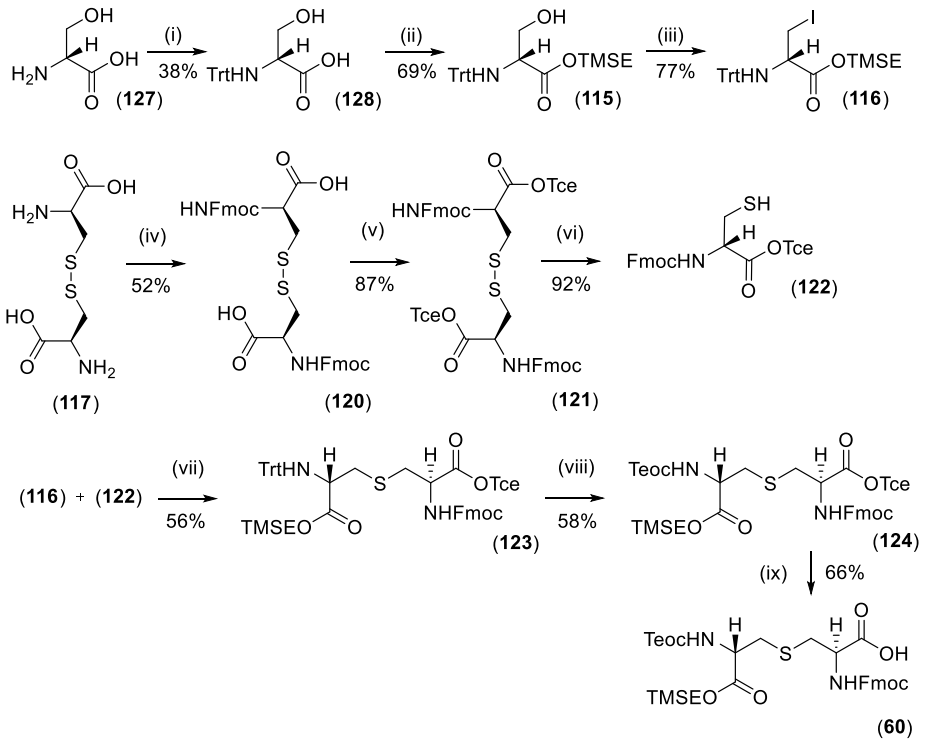


*Scheme 2-9: Fmoc protection of L-cystine (**117**)*

A handful of groups had reported the Fmoc protection of cystine. The conditions of Kirichenko *et al*.[194] were first employed which used sodium carbonate and Fmoc-Cl at -10 °C. Whilst the reaction appeared to go to completion as shown by TLC analysis, problems were encountered during the workup. Despite reporting to require only a simple filtration step, a 5% yield was obtained. Boger *et al*.[195] had previously protected cystine using sodium hydroxide and Fmoc-Cl in THF and water and here the procedure was employed successfully (*Scheme 2-9*). After TLC analysis indicated the reaction had gone to completion THF was removed *in vacuo* and the reaction mixture was acidified using 1.0M HCl. The mixture could then be filtered and the collected solid was re-suspended in ethyl acetate

and left in the fridge overnight. This mixture was again filtered and washed with further portions of cold ethyl acetate to give clean Fmoc protected cystine (**120**) as analysed by NMR.

Tce protection and coupling and replacement of the trityl group was carried out as before. When the final deprotection was carried out on a larger scale it was thought that the final product (**60**) could be purified by recrystallization and several conditions were attempted to avoid a lengthy flash column chromatography purification but these were not successful.



Scheme 2-10: Optimised route for the medium scale synthesis of lanthionine
Reagents and conditions: (i) 1. Trimethylsilyl chloride, 2. MeOH 3. NEt_3, Ph_3CCl (ii) TMSEOH, DMAP, DCC (iii) PPh_3, DEAD, MeI, -10 °C (iv) NaOH, Fmoc-Cl (v) pTSA, TceOH, *100 °C, (vi) DTT, NEt_3 (vii) Cs_2CO_3, 0 °C (viii) 1. 10% TFA, 5% TES, 2. $NaHCO_3$, Teoc-ONSu, (ix) NH_4OAc, Zn dust*

This route was carried out on scales up to 190 mmol, 25 g of starting materials, with an overall yield of 9% from cystine (**117**) and 4% from D-serine (**127**).

2.3 Summary

Orthogonally protected lanthionine (**60**) was initially synthesised using a previously published route on a small scale to give 40 mg of protected lanthionine ready for

incorporation into ring AB analogues of nisin with an overall yield of 5% from Boc-D-Ser(Bn) (**112**) and 10% from (Fmoc-Cys-O^{t}Bu)$_2$ (**119**). To enable large scale synthesis, adjustments to the reactions conditions and purification procedures were required. Flash column chromatography was avoided in two steps which made the route to intermediate cysteine (**122**) more viable for medium and large scale synthesis and increased the overall yield from (Fmoc-Cys-O^{t}Bu)$_2$ (**119**) to 17%. This was carried out on 20-25 g of starting materials to give about ~250 mg of lanthionine (**60**).

Investigations into the use of cheaper starting materials proved successful. The number of steps to protected lanthionine were significantly reduced, decreasing the time required for synthesis and avoiding the potentially hazardous steps of large scale hydrogenation and use of perchloric acid whilst also using significantly cheaper starting materials. This route was also carried out using 20-25 g of starting materials to give 350 mg of lanthionine (**60**) with an overall yield of 9% from cystine (**117**) and 4% from D-serine (**127**) with room for further optimisation. This is a significant improvement from the previously used route of Mothia which had overall yields of <1% from Boc-D-Ser(Bn) (**112**) and 1% from cystine (**117**).

3 Synthesis and Preparation of WT Rings AB of Nisin and Analogues

This chapter describes the SPPS of simplified analogues of rings AB (residues 1-12) and the preparation and purification of WT rings AB from a commercial preparation of nisin. These are intended for use in lipid II binding studies and in hybrid lantibiotics with simpler, linear, pore forming peptides.

3.1 Target Analogues

To simplify the structure of rings AB, all analogues (**129**-**131**, *Figure 3-1*) were designed to contain only lanthionine in comparison to WT rings AB (**93**) which contains both lanthionine and methyllanthionine. It was anticipated that this modification would be well tolerated due to the nature interaction between lipid II and rings AB as it has been shown to rely on hydrogen bonding of the backbone amides.[125] In addition, mutacin I, a structurally related analogue of nisin contains only lanthionine in its AB ring system and shows good binding to lipid II suggesting methyllanthionine is not essential for activity.[196]

The analogues were simplified further by replacing the dehydro residues of WT rings AB with more stable residues. There is good literature precedence to suggest these substitutions would have minimal impact on the binding of rings AB to lipid II: random mutagenesis studies of rings AB suggested modifications of ring A are well tolerated;[197] Chan *et al.*[93] were able to isolate a mutant of nisin with Dha5 substituted with alanine which had little impact on the activity inhibiting growth of *Lactococcus lactis* and *Micrococcus luteus*; Dhb2 of nisin z was replaced with Ser, Ala and Val and the mutations were found to have little impact on the MIC (all remained under 5 nM compared to nisin, 3.3 nM) with only slightly reduced activity against lipid II-containing liposomes.[198]

The first target analogue (**129**) was designed using the biosynthetic precursors, serine and threonine, as mimics for dehydroalanine and dehydrobutyrine respectively. Serine and threonine offer some improvement in stability and, as commercially available residues, are significantly easier to incorporate by SPPS into nisin ring analogues than their dehydrated counterparts. As polar amino acids replacing hydrophobic amino acids it is likely they would also increase the aqueous solubility of rings AB and any hybrid peptides the fragment is incorporated into.

The second analogue (**130**) was designed with the saturated counterparts, alanine and aminobutyric acid (Abu), replacing dehydroalanine and dehydrobutyrine which are again, much easier to incorporate and would significantly improve the stability of rings AB.

Target analogue (**131**) was designed to incorporate α-Aminoisobutyric acid (Aib), a known α-helix inducer and stabiliser[199] which is found in several membrane active peptides such as alamethicin.[200] Increased helicity is attributed to the restriction in the range of accessible backbone conformations of the peptide and it would be interesting to see what effect this residue would have on the conformation of rings AB and the impact on binding to lipid II.

Finally, it was necessary to obtain WT rings AB (**93**) from the enrichment of crude nisin and trypsin digestion as described by Slootweg *et al*.[180] This will be required as a control for both lipid II binding studies and in hybrid peptides.

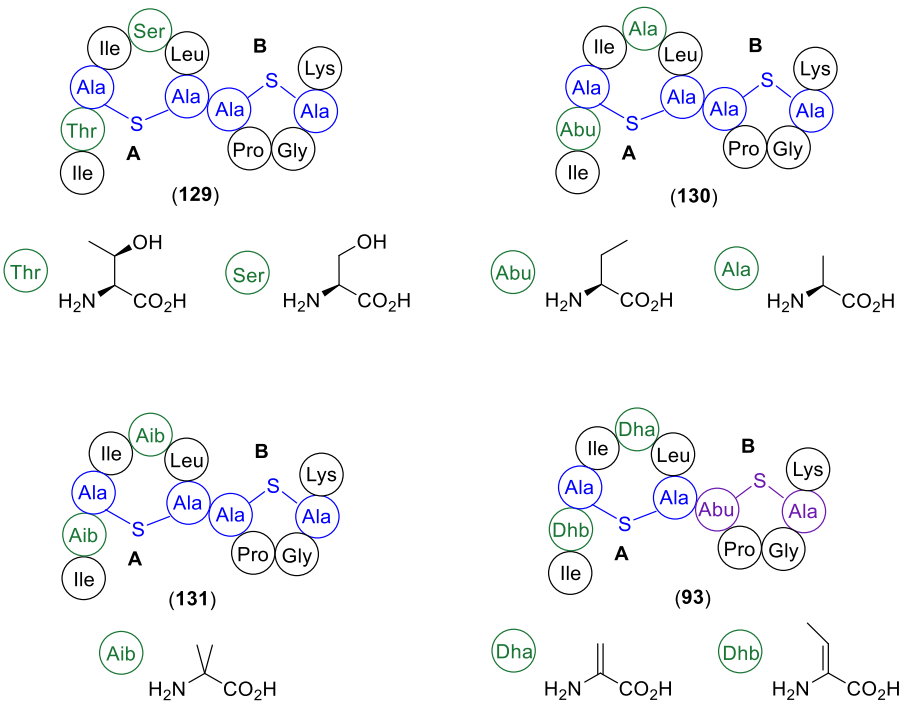


Figure 3-1: Target analogues of rings AB and WT rings AB of nisin

3.2 Peptide Synthesis

Standard Fmoc based SPPS protocol was used throughout peptide synthesis with Nα-Fmoc protected amino acids and permanent Boc/^{t}Bu protecting groups. Fmoc deprotection was carried out with 40% piperidine, and uronium based HOAt and PyAOP were used as coupling reagents with DIPEA as a base as they have been reported to ensure high yielding reactions and reduce racemisation during coupling and cyclisation.[201,202]

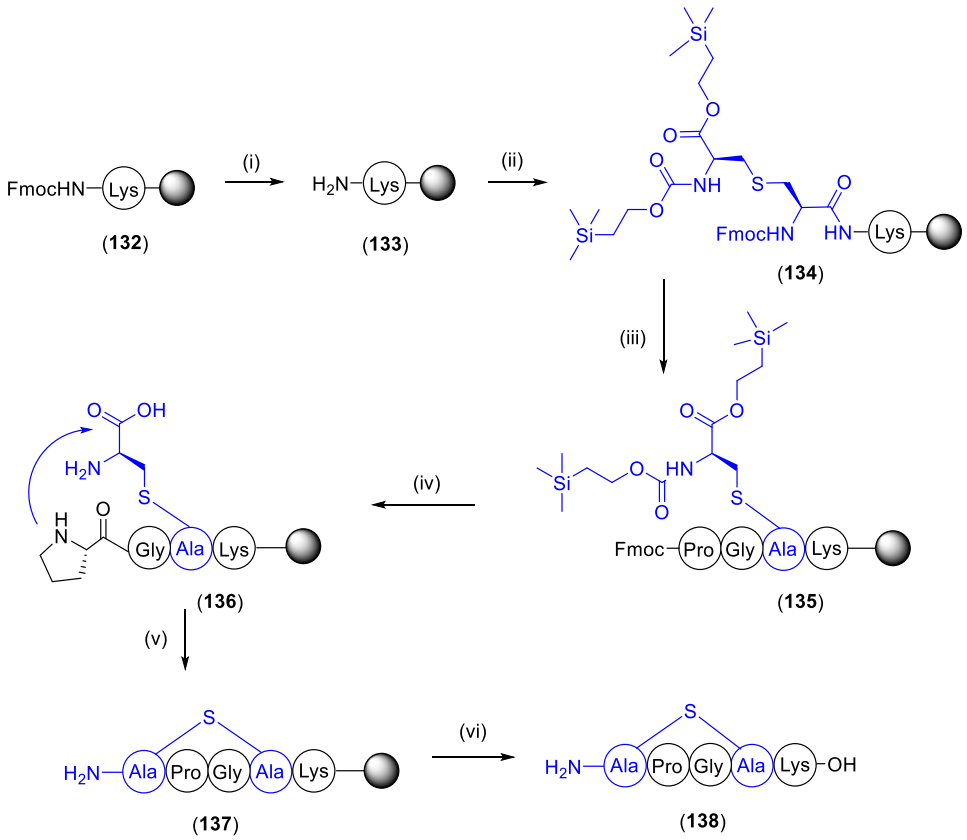
A low loading resin, Fmoc-Lys(Boc)-NovaSyn, 0.20 mmol g^{-1} was used to avoid cross-linking between two complementary functional groups of different peptides on the same resin during the cyclisation, effectively reducing the local concentration. The low loading also helps prevents aggregation of the peptide during the synthesis. Reverse phase high performance liquid chromatography (HPLC) was used for purification of all peptides.

3.3 Ring B Analogue (138)

Before attempting synthesis of the double ring analogues (**129**-**131**) the synthesis of ring B was first attempted to check the SPPS protocol (*Scheme 3-1*).

Synthesis began by first deprotecting the pre-loaded, Fmoc protected residue (**132**) using a solution of 40% piperidine in DMF. After every Fmoc deprotection the resin was manually washed with 6 fresh aliquots of DMF. Washing was followed by coupling orthogonally protected lanthionine residue (**60**) which had been "pre activated" by dissolving in a solution of DIPEA, HOAt and PyOAP in DMF and left for 5 min before addition to the resin. Couplings were left for two hours to ensure the reaction had gone to completion after which the syringe was evacuated and the resin was washed with 4 fresh aliquots of DMF. Fmoc deprotection of (**134**) was carried out as before and this was followed by coupling Fmoc-Gly-OH. Sequential Fmoc deprotection and proline coupling gave linear peptide (**135**). The resin bound intermediate (**135**) was then treated with TBAF to remove the silyl groups of the lanthionine residue. The Fmoc group was likely to be removed by TBAF but Fmoc removal was also carried out to ensure complete deprotection. Cyclisation to form resin bound ring B (**137**) was carried out using the standard coupling conditions of DIPEA, HOAt and PyAOP. The peptide was then cleaved from the resin using a standard TFA cleavage procedure to give (**138**). The crude peptide was purified using reverse phase HPLC and was obtained with a 4% yield. The low yield was attributed to difficulties carrying out

purification on a small scale as the crude HPLC chromatogram looked reasonable at this stage (*Figure 3-2*).



*Scheme 3-1: Synthesis of ring B of nisin (**138**)*
Reagents and conditions: *(i) 40% piperidine in DMF (ii) incorporation of lanthionine (**60**) with HOAt, PyAOP, DIPEA (iii) standard Fmoc SPPS protocol using HOAt, PyAOP as activators to build up chain (iv) TBAF followed by 40% piperidine (v) HOAt, PyAOP, DIPEA (vi) standard TFA cleavage procedure*

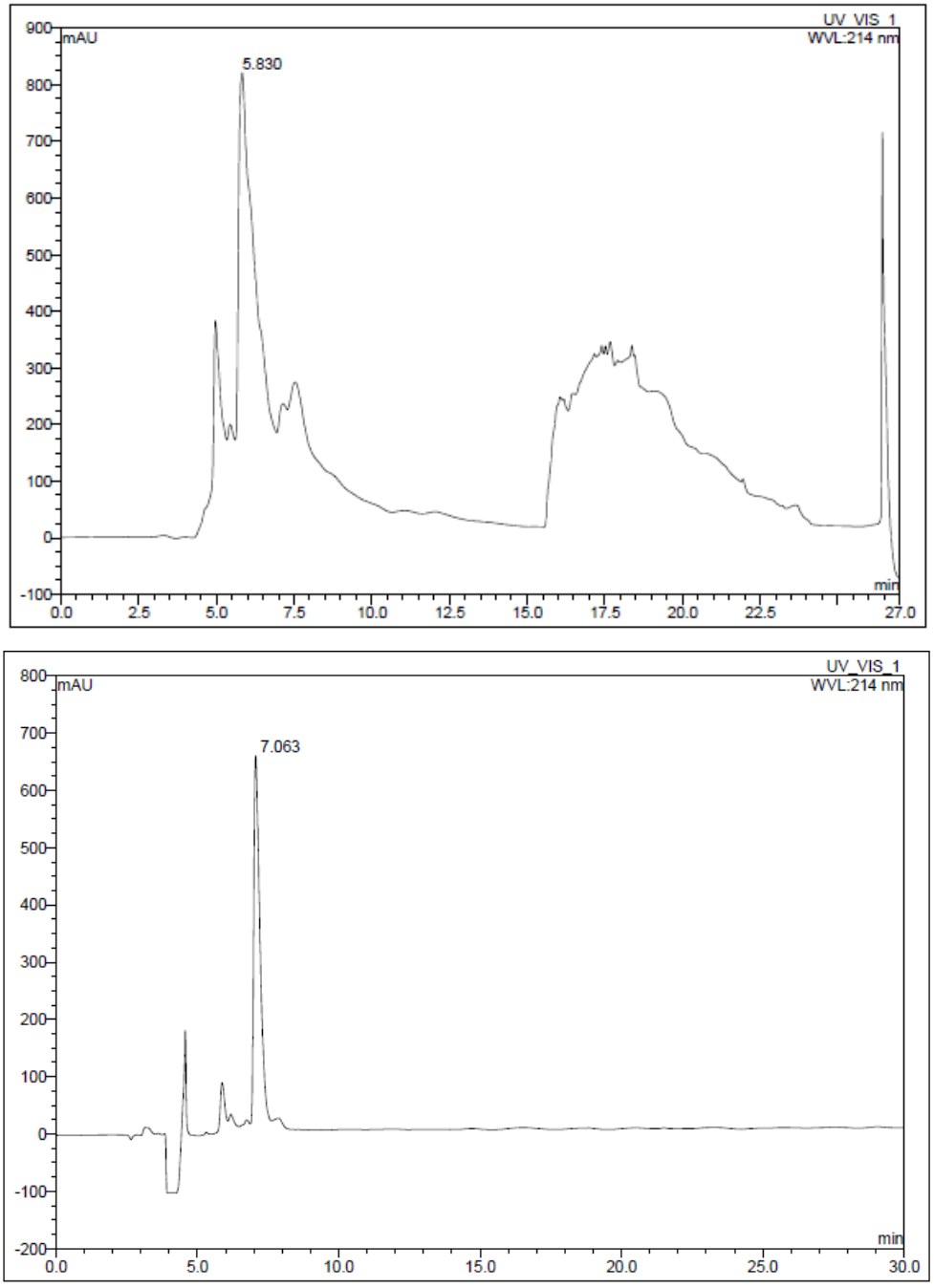
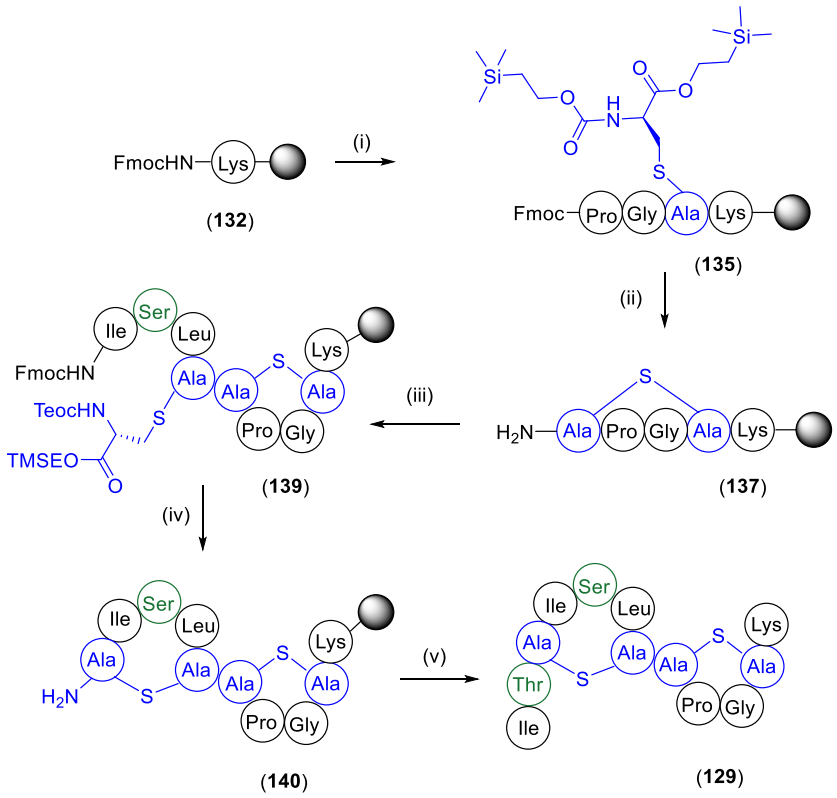


Figure 3-2: Crude HPLC chromatogram of ring B (R_t = 5.83 min); (top) Purified ring B HPLC chromatogram (R_t = 7.06) (bottom)

3.4 Thr/Ser Analogue (129)

With ring B analogue (**138**) in hand, the synthesis of the Thr/Ser analogue (**129**) was attempted (*Scheme 3-2*). Synthesis began by repeating the protocol used for ring B, however, instead of cleaving resin bound intermediate (**137**) the peptide chain was extended with a second lanthionine residue. Sequential Fmoc deprotections and couplings

of leucine, serine and isoleucine were carried out and then followed by a TBAF deprotection of TMSE/Teoc and an Fmoc deprotection of intermediate (**139**). A second intramolecular cyclisation was carried out and then the last two residues threonine and isoleucine were coupled (**140)**. The peptide was cleaved from the resin using a standard TFA cleavage procedure.

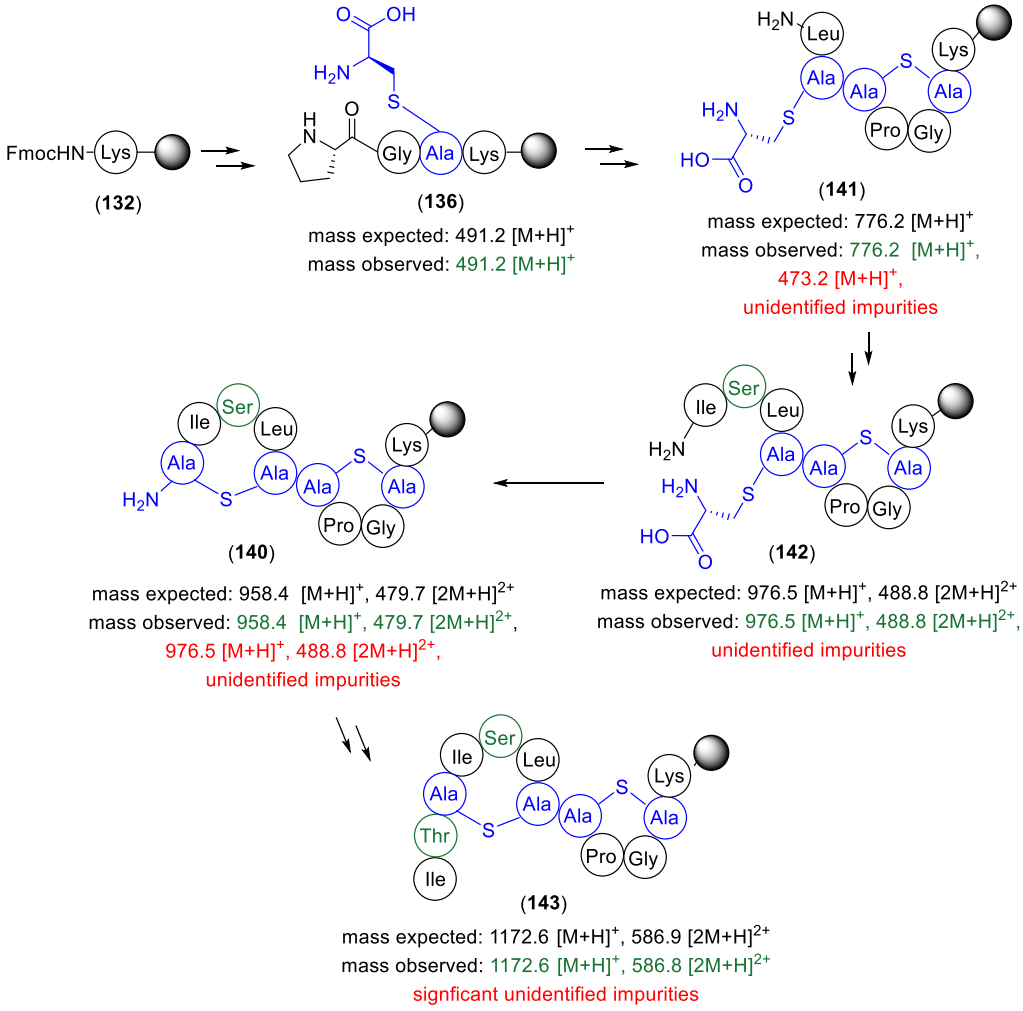


*Scheme 3-2: Synthesis of Thr/Ser analogue (**129**)*
*Reagents and conditions: (i) standard Fmoc SPPS protocol using HOAt, PyAOP as activators to build up chain (ii) TBAF followed by 40% piperidine and then HOAt, PyAOP, DIPEA (iii) standard Fmoc SPPS protocol to incorporate lanthionine (**60**) and to build up chain (iv) TBAF followed by 40% piperidine and then HOAt, PyAOP, DIPEA (v) standard Fmoc SPPS to extend chain followed by standard TFA cleavage procedure*

Using this protocol, product (**129**) was visible by LCMS but a significant number of impurities were also present and it was not possible to isolate the product *via* reverse phase HPLC due to large amounts of impurities.

To assess where impurities were being introduced the synthesis was repeated. Periodically, small batches of resin (around 2 mg) were taken from the syringe and were treated with cleavage solution (*Scheme 3-3*). This was then filtered, diluted with water and used directly for crude MS analysis to record the progression of the peptide synthesis. Although this procedure was useful in identifying problems with the synthesis it was not reliable and

sometimes none of the expected masses could be observed. This was possibly due to ion suppression caused by a high concentration of TFA in the sample which, in future, could be avoided by precipitating the cleaved peptide in ether and analysing the resulting precipitate to avoid excess amounts of TFA.[203] Alongside the identified impurities, significant amounts of unidentified impurities could be seen on the LCMS mass spectra although some of this could be attributed to background noise due to the very low concentration of the samples.



Scheme 3-3: Results of the mini-cleave experiments

Synthesis was shown to proceed successfully until the addition of the second lanthionine residue. A peak for cyclised ring B (**138**) (*m/z* = 473.2) was visible in the proceeding cleavages whereby lanthionine or any of the proceeding residues had failed to couple to the N-terminus of the ring. This suggested that the terminal amine of ring B was likely to be sterically unavailable for further coupling, possibly pointing towards the centre of the ring.

Preliminary NMR work carried out by Dickman on (**138**) and WT nisin ring B has supported this idea, showing that the proline amide bond sits in a *cis* conformation which can cause the terminal amine to point towards the middle of the ring.[204] The additional steric bulk of lanthionine could have also contributed to poor rates of reaction for this step.

The second significant problem encountered was the cyclisation of ring A. Here the MS analysis showed a peak for both cyclised (**140**) and uncyclised peptide (**142**) (*m/z* = 976.8) suggesting cyclisation had not gone to completion (*Figure 3-3*). This was likely to be due to the failure of the uncyclized intermediate to pre-organise in such a way that the amine of Ile was near to the carboxylic group of lanthionine. Following incomplete cyclisation, a significant number of different products could be produced in the proceeding coupling steps through inter- and intra-molecular coupling. Poor rates of coupling and ring closure are problematic in producing impurities and low yields and they will also increase the chances of C-terminal epimerisation.

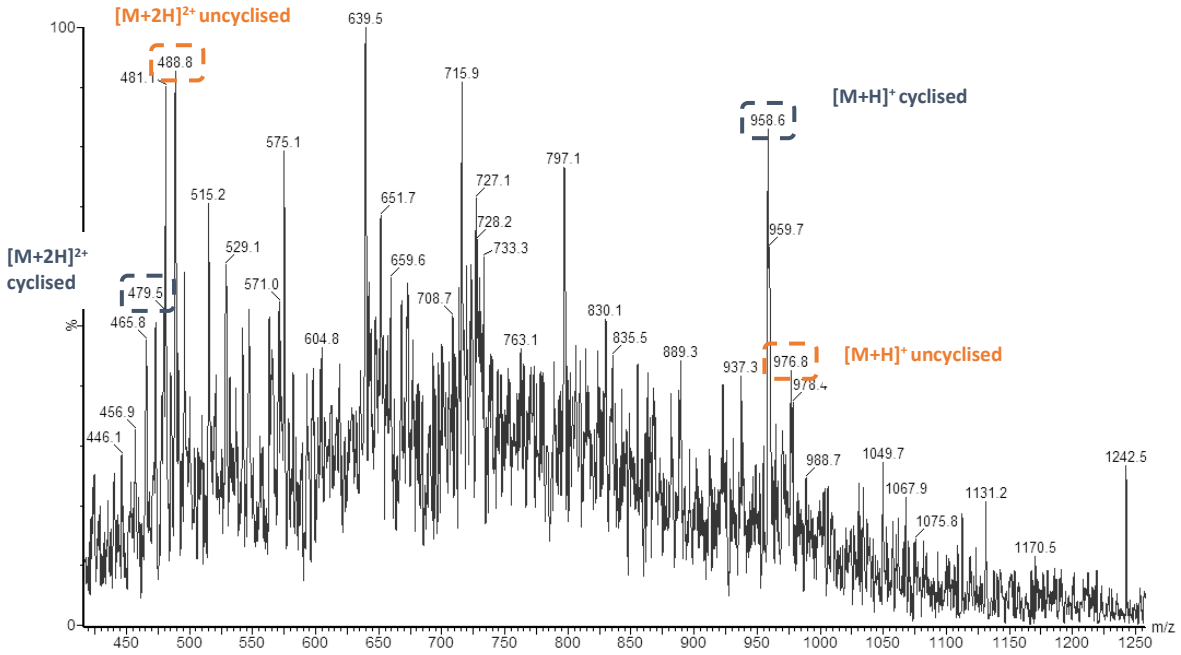


Figure 3-3: LRMS mass spectrum of mini cleave experiment taken after cyclisation

Previously within the group, Wright, had shown microwave conditions improved the coupling of lanthionine to a linear peptide chain[190] and it was thought that these conditions could also improve the coupling of the second lanthionine in the ring AB analogue (**129**).

Performed first in a modified kitchen microwave in 1991,[205] microwave assisted SPPS is now fairly commonplace with purpose built microwave technology incorporated into bench top automated peptide synthesisers. Microwave irradiation is beneficial to SPPS in two ways: rate of reactions can be increased significantly which improves purity of crude peptides and this also cuts the reaction time down, decreasing the need for multiple couplings or deprotections. This can be especially useful for the coupling of bulky amino acids.[206] The elevated temperatures have also been shown to increase racemisation and so it is recommended to decrease reaction temperatures to 50 °C for histidine and cysteine.[207]

Wright reported optimised conditions of 5 minutes and 60 °C with longer reaction times and higher temperatures having a detrimental effect on yield. Indeed, when microwave conditions were used for every coupling and cyclisation step during the synthesis of rings AB (**129**) no product could be observed in the crude LCMS (*Table 3-1*). Microwave conditions were then limited to installing both lanthionine residues.

To help each coupling step to completion double coupling of every residue apart from lanthionine was employed as standard throughout the rest of the ring AB analogues syntheses. This is a commonly used technique in SPPS for the synthesis of difficult sequences whereby a second solution of the required amino acid and coupling agents are added to the syringe after the first solution has been evacuated.[208] The limited availability of lanthionine meant this was not practical for the lanthionine coupling steps.

Conditions	**Product present in LCMS**	**Product isolated *via* HPLC**
Single couplings of all residues, no microwave, 5 equivalents of lanthionine	Yes	No
Single couplings of all residues with microwave conditions for every step, 5 equivalents of lanthionine	No	-
Double couplings of all residues apart from lanthionine, coupled with 3 equivalents and microwave conditions	Yes	No

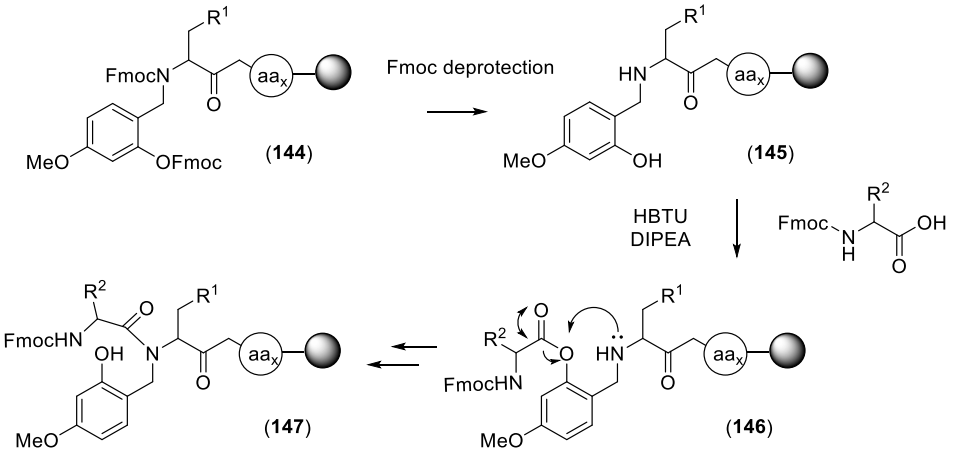
*Table 3-1: Conditions used for the attempted synthesis of ring AB analogue (**129**)*

Unfortunately, in this case, double coupling and microwave conditions did not improve the synthesis of rings AB (**129**) enough to enable purification and isolation of the target

compound. However, microwave conditions did allow a decrease in the equivalents of lanthionine required for coupling and so further synthesis was carried out using 3 equivalents of lanthionine instead of 5.

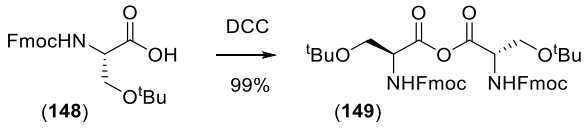
3.4.1 Use of Hmb-Protected Amino Acids

Again, looking back to previous work carried out by Wright, Hmb-protected amino acids were used to aid the synthesis of large lanthionine-containing rings where it was suspected that aggregation was disrupting coupling of amino acids within the ring system. Whilst Hmb-protected amino acids are widely used for this purpose as they disrupt hydrogen bonding of the backbone, it was thought that in the smaller ring systems of AB where aggregation is unlikely to be occurring, the addition of N-alkylated residues may alter the uncyclised solution structure in such a way that cyclisation is favoured. On searching the literature, it was found that N-alkylation does have an impact on the conformation of small peptide rings[209] and both N-methylation and Hmb protected amino acids had been used to improve cyclisation of pentapeptides.[210]



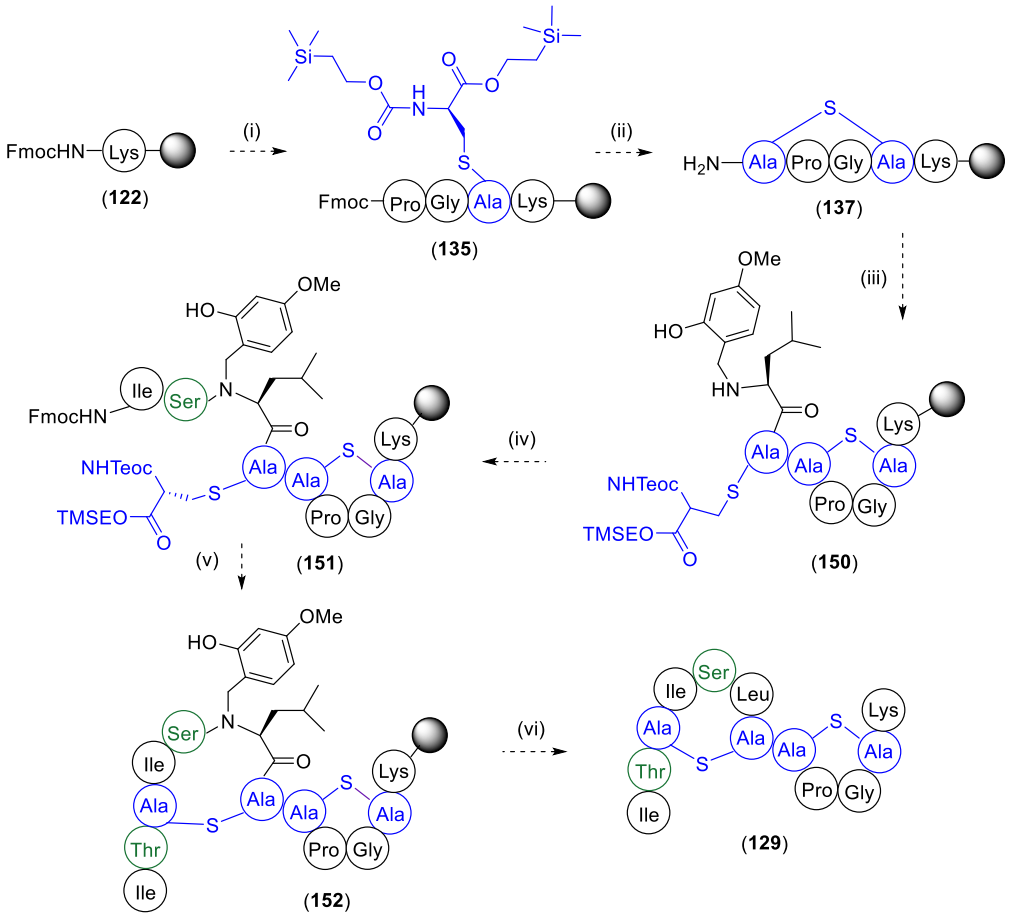
Scheme 3-4: Mechanism of acetyl transfer of Hmb residues

The bulky nature of the Hmb group means that coupling of the proceeding residue can be disturbed and it is understood that the residue reacts first with the aryl alcohol of the Hmb group (*Scheme 3-4*). This enables acyl capture to form intermediate (**146**), followed by intramolecular acyl transfer to give the desired product (**147**). Peptide synthesis can then continue using the standard Fmoc SPPS protocol to build up the chain and the Hmb group is removed during the standard acidic conditions used to cleave the resin. Literature protocols suggest the most successful way to couple the proceeding amino acid is to use the symmetric anhydride of the required amino acid (*Scheme 3-5*).[211]



Scheme 3-5: Synthesis of the symmetrical anhydride of serine

Hmb-Leu was used at position 7 in ring A to see if this would help cyclisation (*Scheme 3-6*). The symmetrical anhydride of serine was synthesised using DCC as a coupling agent (**149**, *Scheme 3-5*). The reaction mixture was filtered and the product was easily identified by a shift of the carbonyl carbon signal in the ^{13}CNMR. The symmetric anhydride of serine was carried forward crude and coupled to the peptide chain using literature conditions[211] with 10 equivalents in CH_2Cl_2. The rest of the synthesis was carried out as before but unfortunately a signal for the desired product could not be seen in the crude LCMS.



*Scheme 3-6: Unsuccessful synthesis of analogue (**129**) using Hmb-Leu*
Reagents and conditions: (i) standard Fmoc SPPS protocol using HOAt, PyAOP as activators to build up chain (ii) TBAF followed by 40% piperidine and then HOAt, PyAOP, DIPEA (iii) incorporation of Hmb-Leu using HBTU, DIPEA followed by 40% piperidine in DMF (iv) incorporation of serine symmetric anhydride in CH_2Cl_2 followed by standard Fmoc SPPS protocol to build up chain (v) *TBAF followed by 40% piperidine and then HOAt, PyAOP, DIPEA followed by standard Fmoc SPPS protocol to build up chain (vi) standard TFA cleavage procedure*

3.4.2 Use of Pseudoprolines

Hmb protected amino acids belong to a group of commercially available, reversibly Nα-alkylated amino acids designed to disrupt formation of secondary structures and aggregation of growing peptide chains in SPPS. Another approach to this problem is the use of pseudoprolines, in which cysteine, threonine and serine derived dipeptides are reversibly protected with proline-like thiazolidines or oxazolidines which are returned to their respective derivatives on cleavage with standard acid conditions (*Figure 3-4*).[212] These are incorporated as dipeptides and are increasingly commercially available. It has also been reported that they have aided in the head-to-tail cyclisation of both short[213] and longer[214] peptides. This effect can be attributed to the observation that they induce a predominantly

cisoid conformation about the amide bond adjacent to the modified amino acid, resulting in the temporary induction of a β-turn much like proline is found to do. It was thought that incorporating a pseudoproline in ring A could help ring closure proceed to completion, essentially, pre-organising the peptide for ring closure.

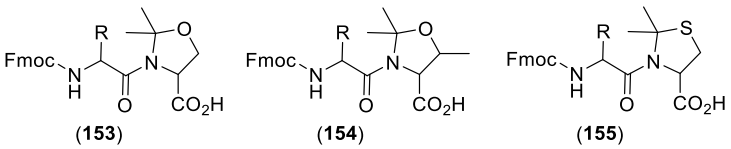
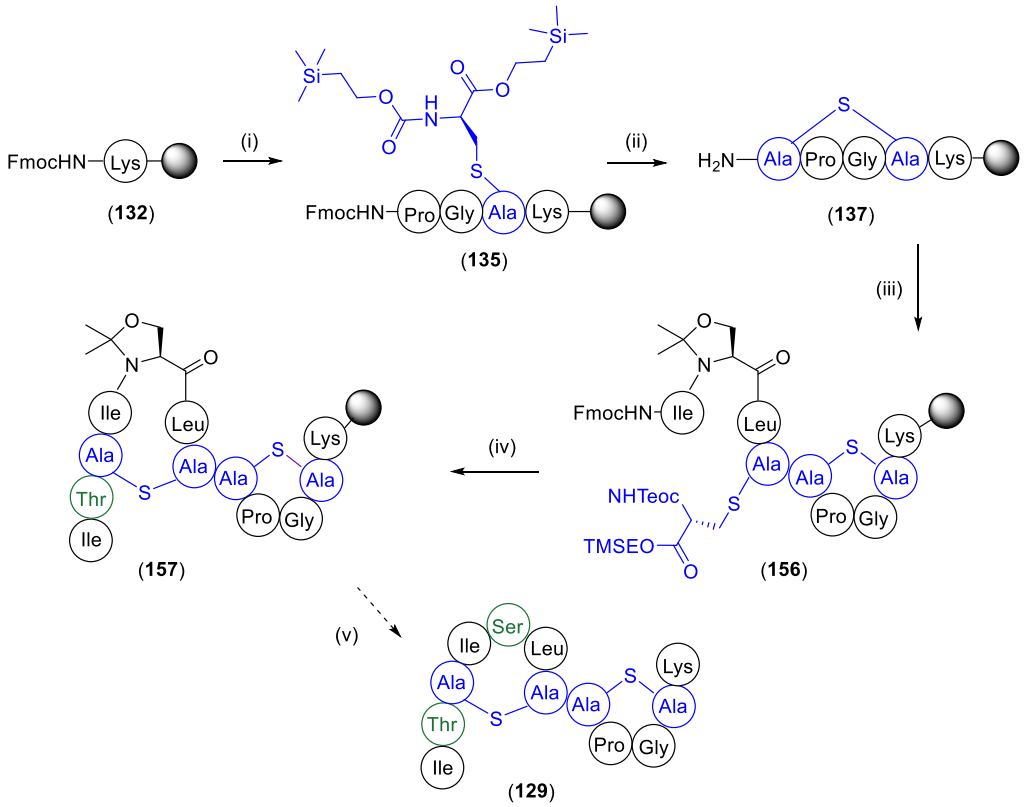


*Figure 3-4: Serine (**153**), threonine (**154**) and cysteine (**155**) derived pseudoprolines*

Fortunately, the combination of ring A containing a serine residue and the commercial availability of Fmoc-Ile-Ser[ψ(^{Me,Me})pro]-OH meant a pseudoproline residue could be easily incorporated into ring A of Thr/Ser analogue (**129**). Pseudoproline residues also provide the advantage of incorporating two residues at once, reducing reaction times and the introduction of impurities.

Fmoc-Ile-Ser[ψ(^{Me,Me})pro]-OH was coupled onto intermediate (**125**) using the same conditions as all other residues, double coupling with 5 equivalents of amino acid (*Scheme 3-7*). The sequence was completed as before and cleaved from the resin using a standard TFA cleavage procedure.



*Scheme 3-7: Attempted synthesis of rings AB (**129**) using pseudoproline*
Reagents and conditions: (i) standard Fmoc SPPS protocol using HOAt, PyAOP as activators to build up chain (ii) TBAF followed by 40% piperidine and then HOAt, PyAOP, DIPEA (iii) incorporation of Fmoc-Leu-OH and Fmoc-Ile-Ser[ψ(^{Me,Me})pro]-using standard Fmoc SPPS protocol (iv) TBAF followed by 40% piperidine and then HOAt, PyAOP, DIPEA followed by standard Fmoc SPPS protocol to extend chain (v) standard TFA cleavage procedure

Crude LCMS analysis looked promising showing one significant product. However, the main product observed was not the target product but the protected pseudoproline derivative (**158**) *m/z* = 607.2 *(Figure 3-5*).

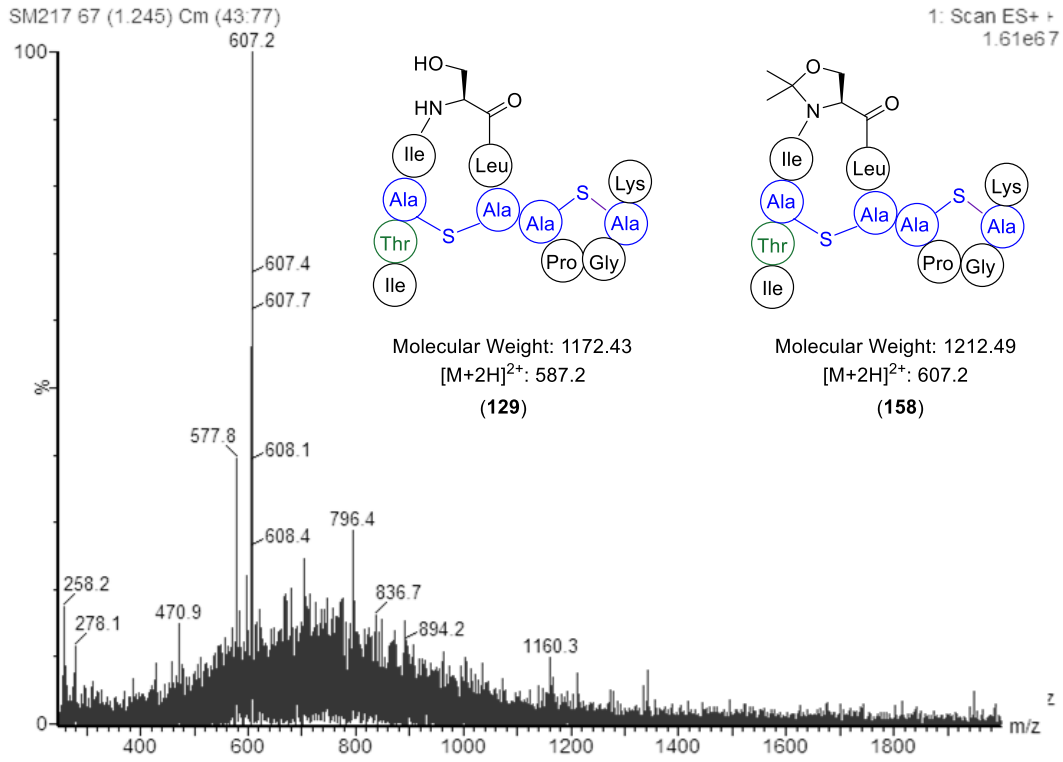


*Figure 3-5: Crude mass spectrum of attempted synthesis of (**129**) showing pseudoproline protected (**158**)*

Surprisingly, the acidolysis of the ring had not occurred in aqueous 95% TFA over 40 mins. On searching the literature, it was found that several other groups had experienced difficulty removing threonine derived pseudoprolines of cyclic peptides and these had required long deprotection times up to 4 days.[215–217] It is also established that the cysteine derivatives are much more stable often requiring long reaction times or very strong acids like triflic acid (TFMSA).[212]

Increasing the reaction time for the deprotection of (**158**) with TFA did increase the amount of desired product (**129**) as visible by LCMS analysis but also visible were degradation products. Different solvent systems using 10% CH_2Cl_2 and 10% water in TFA were also investigated but these did not help the rate of deprotection (*Table 3-2*).

Returning to the literature, Wong *et al.*[215] had incorporated Cys($\psi^{Me,Me}$pro) into the sequence of cycloglossine B to aid cyclisation and found that the deprotection of the thiazolidine required 15 minutes at 0 °C with 100% TFMSA to obtain complete deprotection. The product was precipitated using diethyl ether in the same way as is standardly used to remove TFA cleavage solutions. Using this procedure, deprotection was

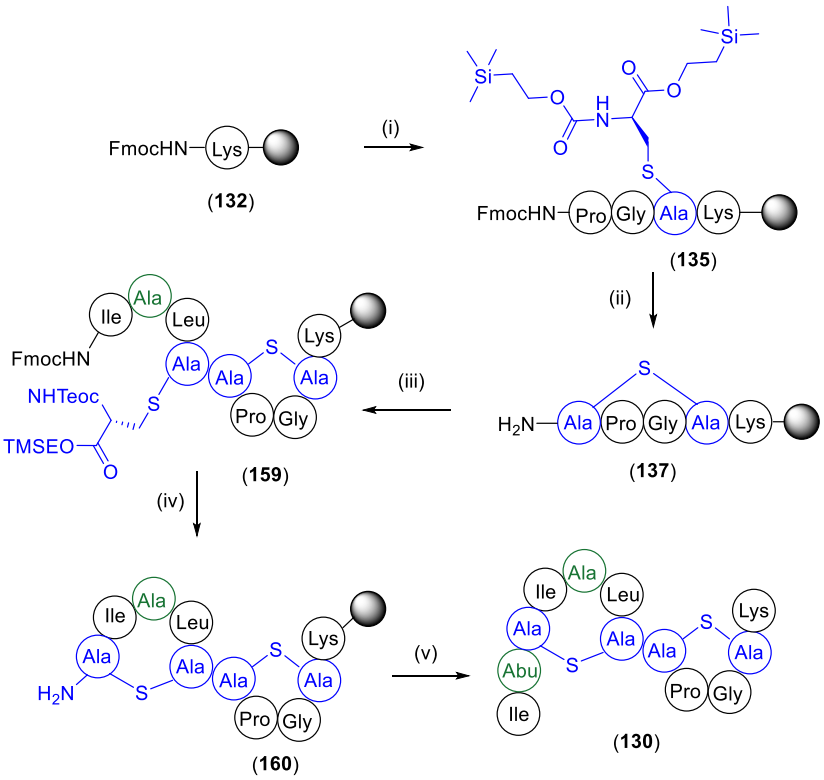
shown to go to completion by LCMS analysis. A shorter reaction time of five minutes was then trialled and complete deprotection was also observed. An overall yield of 200 µg, 1% was obtained for rings AB (**129**) after reverse phase HPLC.

Conditions	Time	Results
96.5% TFA, 2.5% H_2O, 1% TIPS	40 mins	No deprotection
90% TFA, 10% H_2O	24 hours	Partial deprotection & degradation products
90% TFA, 10% CH_2Cl_2	24 hours	Partial deprotection & degradation products
100% TFMSA, 0 °C	15 mins	Complete deprotection
100% TFMSA, 0 °C	5 mins	Complete deprotection

*Table 3-2: Summary of conditions used to deprotect (**158**)*

3.5 Abu/Ala Analogue (130)

The alkyl analogue (**130**) was synthesised using the same procedure as had been used for Thr/Ser analogue (**129**) with double coupling and microwave conditions for the incorporation of lanthionine (*Scheme 3-8*). In this case the product could be purified from the crude mixture to give an overall yield of 1%.



*Scheme 3-8: Synthesis of Abu/Ala Analogue (**130**)*
*Reagents and conditions: (i) standard Fmoc SPPS protocol using HOAt, PyAOP as activators to build up chain (ii) TBAF followed by 40% piperidine and then HOAt, PyAOP, DIPEA (iii) standard Fmoc SPPS protocol to incorporate lanthionine (**60**) and to build up chain (iv) TBAF followed by 40% piperidine and then HOAt, PyAOP, DIPEA (v) standard Fmoc SPPS protocol to extend chain followed by standard TFA cleavage procedure*

3.5.1 Use of Hmb-Protected Amino acids

To improve the yield of the Abu/Ala analogue (**130**) two different commercially available, Hmb residues were incorporated into ring A, Hmb-Ala and Hmb-Leu (*Figure 3-6*). The appropriate symmetrical anhydrides were synthesised and incorporated as before after the Hmb residues (*Scheme 3-9*). No product could be seen in the crude LCMS in either case.

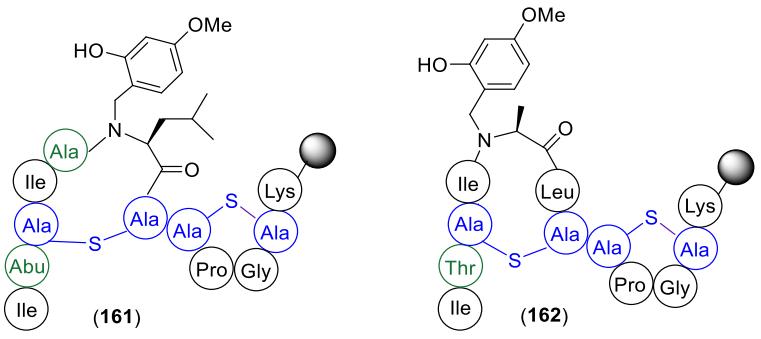
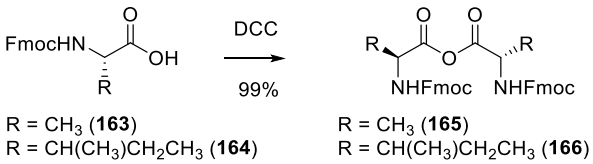


Figure 3-6: Incorporating Hmb residues into ring A



*Scheme 3-9: Synthesis of symmetrical anhydrides of alanine (R=CH_3) (**165**) and isoleucine (R=$CH(CH_3)CH_2CH_3$) (**166**)*

3.6 Aib/Aib Analogue (131)

The synthesis of analogue (**131**) with Aib residues was also attempted using the same procedure as used for the Abu/Ala analogue (**130**) but unfortunately the product was not visible in the crude LCMS.

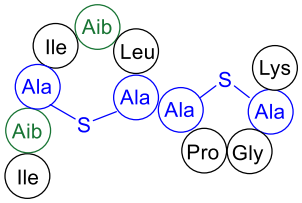


*Figure 3-7: Target Aib/Aib analogue (**131**)*

Two main products were visible in the crude LCMS both visible as $[M+H]^+$ and $[M+2H]^{2+}$ suggesting they were both lysine containing peptides. Tandem mass spectrometry analysis was carried out on the two main components but the results were inconclusive. It is likely the failure was due to the additional bulkiness at the alpha carbon of Aib compared with Ala or Ser. Further work should look at using the established microwave conditions to incorporate Aib. Subiros-Funosas *et.al*.[218] successfully used Oxyma-based uronium-type coupling reagent, COMU, and microwave conditions to improve the synthesis of an Aib containing pentapeptide and these conditions could be worth pursuing for future synthesis.

3.7 Recovering Excess Lanthionine

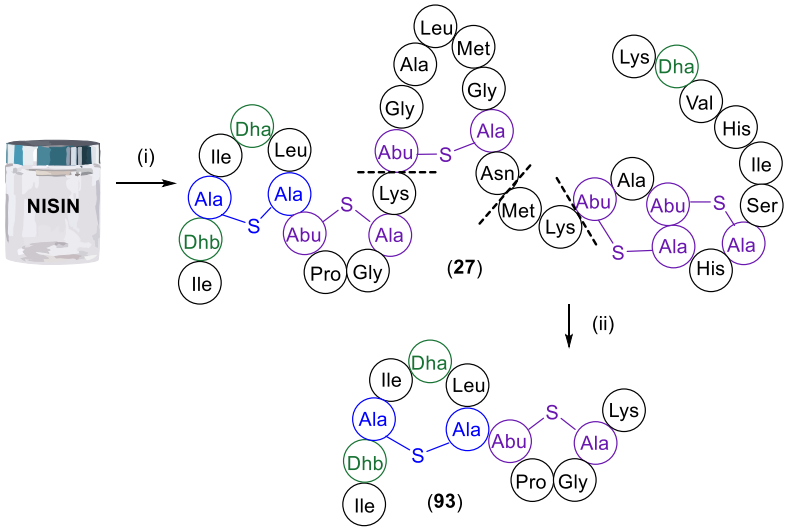
Standard SPPS amino acid coupling procedures use an excess of amino acid to ensure each coupling goes to completion and the excess reagents are easily removed by evacuation through the syringe. This is not an ideal protocol for coupling of modified amino acids of limited quantity such as lanthionine. The equivalents of lanthionine (**90**) were reduced to 3 to minimise waste and a procedure for recovering unreacted lanthionine was investigated.

To remove side products, excess coupling agent and DMF the evacuated coupling solution was washed with a large excess of a solution of 5% citric acid and extracted with CH_2Cl_2. The resulting residue was purified by flash column chromatography to give clean lanthionine in good yield, 60%. Occasionally small amounts of DIPEA were still present after column chromatography and another washing with citric acid was required.

3.8 WT Rings AB (93)

3.8.1 Enrichment of Nisin

WT rings AB of nisin were obtained using a literature procedure outlined by Slootweg *et al.*[180] for the trypsin digest of commercially available WT nisin (*Scheme 3-10*). Typically, commercially available preparations of nisin contain 75% NaCl, 22.5% dairy proteins and only 2.5% of nisin and so first an extraction was carried out, as outlined by Slootweg, to perform the digest on a more enriched sample of nisin. The enrichment was carried out using 25g of commercially available preparation purchased from MP Biomedicals and would give around 600-700 mg of enriched nisin.



*Scheme 3-10: Procedure for obtaining WT rings AB (**93**)*
Reagent and conditions: (i) water, CH_2Cl_2 followed by centrifugation and filtration (ii) tris acetate buffer, trypsin, 30 °C, 40 h

The commercial preparation of nisin was dissolved in water and then CH_2Cl_2 was added to precipitate out the peptide content which formed a cloudy precipitate in the aqueous layer. The solution was then transferred to falcon tubes and centrifuged causing the precipitate to form a solid pellet, partitioned between the two layers. The solid was

collected and dried under reduced pressure to remove any remaining CH_2Cl_2. It was then re-dissolved in deionised H_2O and filtered through a small column of celite to remove any aggregated peptide material. If the filtrate appeared cloudy this filtration step was repeated. The solution was then concentrated *in vacuo* and lyophilised to give an off-white fluffy powder of enriched nisin concentrated by a factor of 6 as estimated by HPLC (*Figure 3-8*).

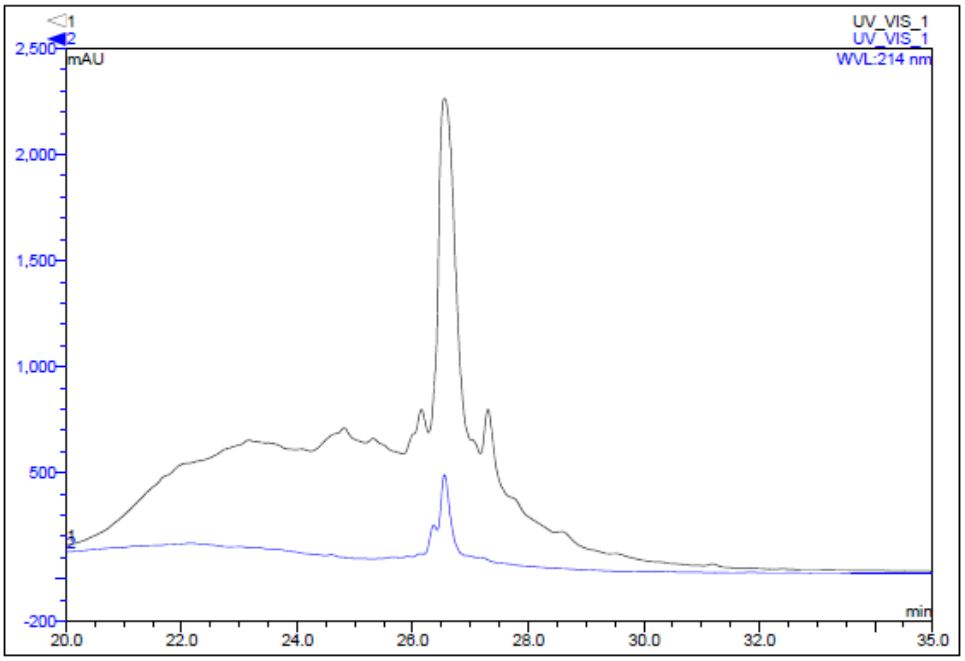


Figure 3-8: HPLC chromatogram of commercially available nisin (bottom trace) and enriched nisin (top trace) at 10 mg mL^{-1}

3.8.2 Trypsin Digest

The enriched nisin was then used without further purification in a trypsin digest according to the published procedure. After HPLC purification 10 mg of rings AB were obtained with estimated 8% yield. Even though the reaction was monitored by HPLC and the digest was shown to go to completion this was significantly lower than the yield obtained in the literature (93%).[180]

Differences in yield may be partially accounted for by the varying quality of commercial sources. Despite using the same conditions, it could be possible that the digested rings AB fragment is being further broken down in the digest being hydrolysed at the dehydro residues.

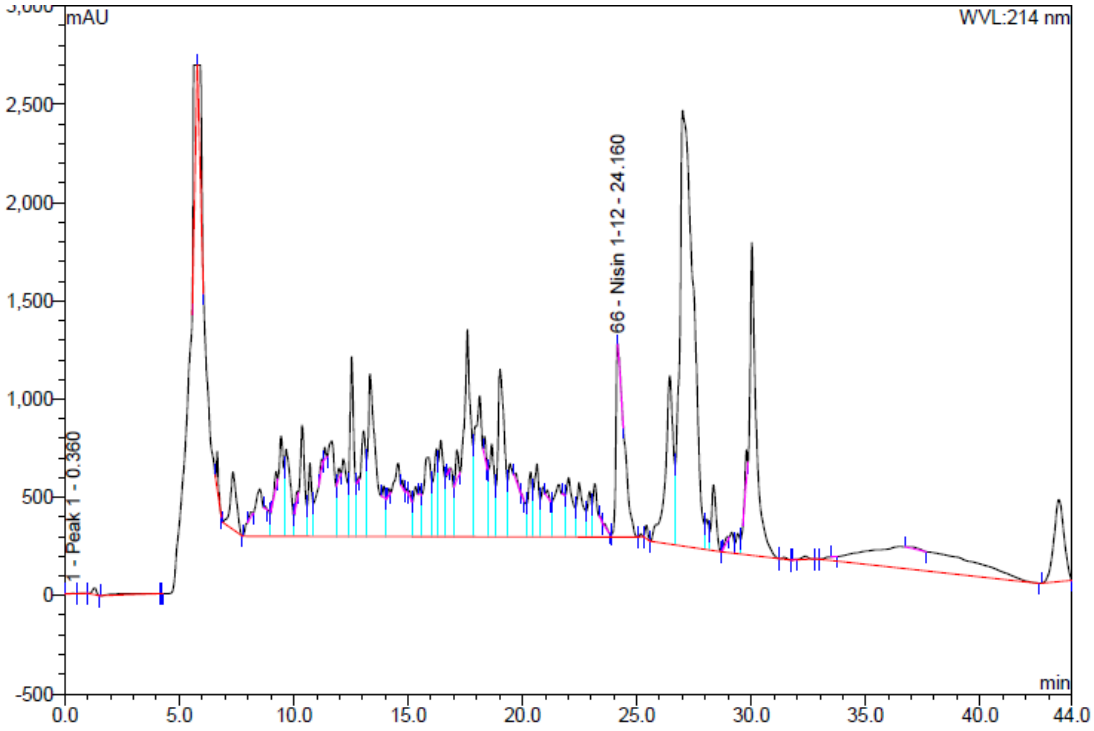


Figure 3-9: Typical HPLC chromatogram for the purification of digested nisin

More recently Koopmans and co-workers also used a trypsin digest on WT nisin using more trypsin and a more concentrated solution of nisin with shorter reaction time.[98] Using these conditions, a slightly improved yield of 11.2% was obtained.

3.9 Summary

The previously published methodology used to synthesise rings DE and rings C of nisin was adapted to enable the synthesis of two novel analogues of rings AB (**129**) and (**130**) and an analogue of ring B (**138**).

Success of SPPS of the ring AB analogues was clearly very sequence dependant with the analogue with the least bulky amino acids proving the most straightforward. Increasing the bulkiness of alanine and aminobutyrine to serine and threonine required a pseudoproline residue to facilitate ring closure which also required an additional deprotection step. The synthesis of analogue (**131**) containing Aib residues was attempted but further work is required to optimise the methodology.

WT rings AB of nisin (**93**) were obtained by using a literature procedure for the enrichment and digestion of commercially available WT nisin. This was intended for use in lipid II binding experiments with the other two AB analogues and used in synthesising hybrid peptides.

4 Hybrid Peptides

With the analogues of nisin rings AB and WT rings AB in hand, the design of the C-terminus tail peptides and hinge region linkers was assessed and the synthesis of hybrid peptides was carried out.

4.1 Hybrid Peptide Design

To enable click chemistry we aimed to attach an azide or alkyne to rings AB and incorporate the corresponding moiety into the hinge region. Coupling an alkyne "handle" to rings AB was chosen due to literature precedence for coupling propargylamine to fragments of nisin as discussed in Chapter 1.4.1.[183,186] The hinge region and tail peptides then needed to incorporate an azide (*Figure 4-1*).

As discussed in Chapter 3.8, multimilligram amounts of WT rings AB were obtained *via* digestion of commercially available nisin. Simplified analogues of rings AB were also obtained *via* SPPS, however, synthesis was complex and low yielding and so hybrids with WT rings AB were first synthesised with the intention of using the simplified SPPS analogues once the methodology had been developed.

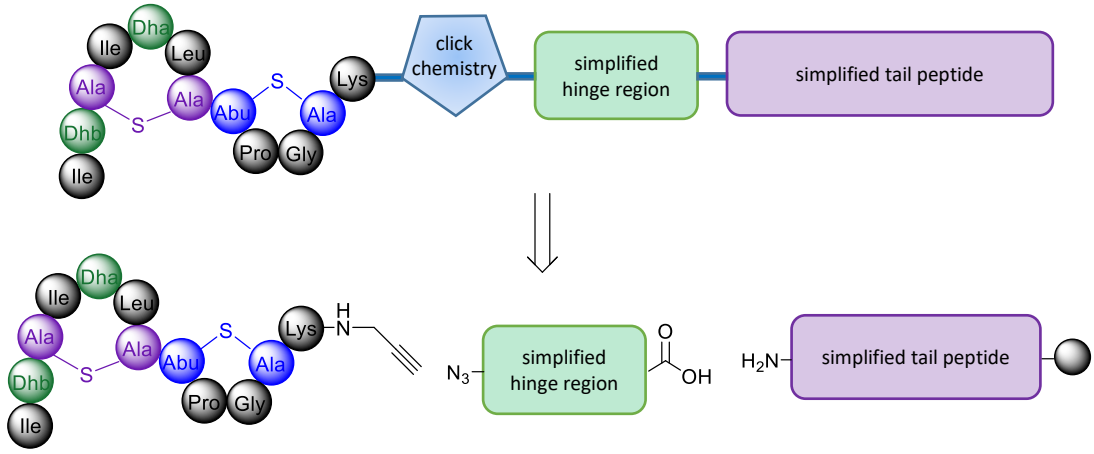


Figure 4-1: Design of hybrid peptides

As previously discussed in Chapter 1.3.8, the hinge region of nisin (residues 20-23) is essential for activity; truncations or mutations to proline, decreasing flexibility, have a negative impact on activity.[164,165] The use of a short PEG chain as a linker is a logical choice due to the commonplace incorporation of PEG into peptides, and other biomolecules, to

aid solubility, maintain flexibility and, for large PEG chains, reduce the reticuloendothelial clearance and recognition by the immune system.[219,220] In addition to the requirement for an azide, the linkers also needed to be easily coupled to the N-terminus of the tail sequences and so they were designed with carboxylic acids to enable easy attachment using standard amide coupling conditions which could be carried out whilst still on resin.

Linear pore forming peptides were chosen to mimic the C-terminus of nisin; we envisaged that replacing rings D and E with non-lanthionine containing peptides would significantly reduce the synthetic challenge of synthesising compounds with nisin's mode of action. A reduction in the number of lanthionine residues also reduces the number of residues prone to oxidation. Cationic sequences were chosen to mimic the polarity of nisin and to enhance aqueous solubility. Removing the thioether bridges may increase the susceptibility of the hybrid peptides to enzymatic degradation and so the use of peptoids as mimics for nisin's tail region was also investigated. These tail segments were synthesised using standard Fmoc SPPS protocol and standard peptoid sub monomer synthesis respectively.

4.2 Tail Peptides

Two peptides with established pore forming, antibacterial activity (as discussed in Chapter 1.1) were chosen for incorporation into the hybrid peptides: a mastoparan analogue, Polybia-MP1 (**12**), and pexiganan (**10**), developed from magainin.[57] The peptoid (**15**) designed by, Patch and Barron,[85] to mimic the helix of magainin 2, was also chosen as a tail segment.

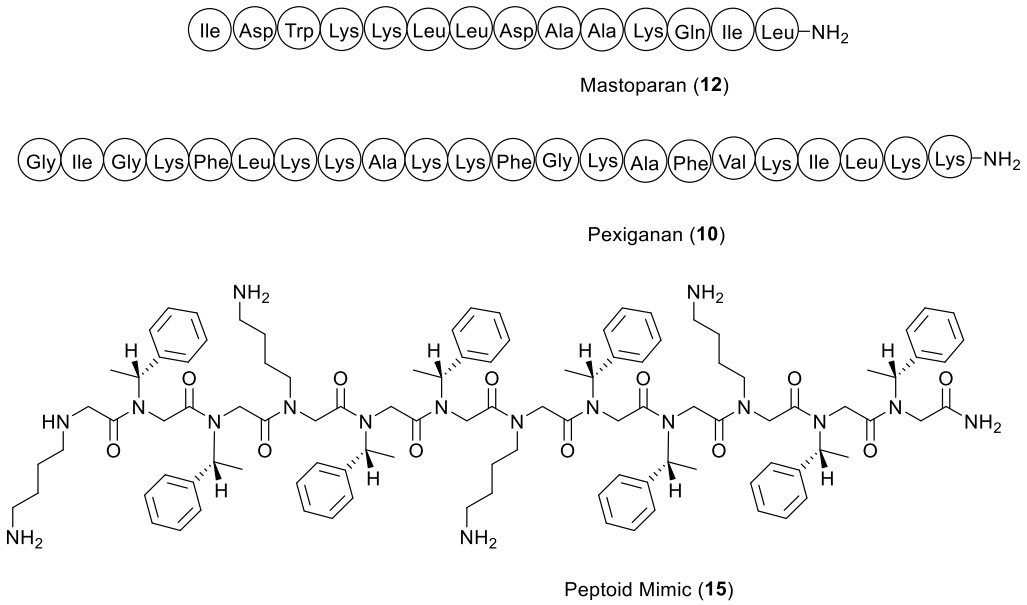


Figure 4-2: Selected "tails" for hybrid peptides

4.2.1 Peptide Synthesis

The tail peptides mastoparan and pexiganan were synthesised using an automated peptide synthesiser with standard Fmoc SPPS protocol using HBTU and DIPEA for amino acid couplings and a piperidine/DMF solution for Fmoc deprotection. Rink amide resin was used for all tail peptides synthesised to give C-terminal amide peptides on cleavage.[221] Initially, the tail peptides without linkers were synthesised to check the automated synthesis protocol and to produce control peptides to be used in future biological assessment (

Figure 4-3).

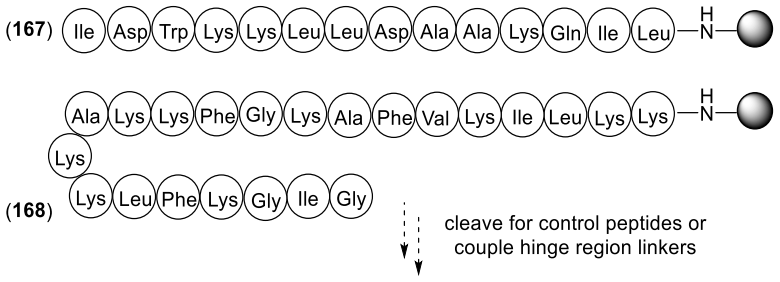


*Figure 4-3: Resin bound intermediates mastoparan (**167**) and pexiganan (**168**)*

The crude LCMS mass spectra of pexiganan (**10**) showed good purity and the peptide was purified by reverse phase HPLC (*Figure 4-6*). A deletion sequence was visible in the crude

LCMS of mastoparan (**12**) whereby the terminal amino acid did not couple onto the N-terminus and several unidentified impurities were also present (*Figure 4-4*). The synthesiser was programmed to carry out double coupling of the amino acids and this significantly increased the purity as analysed by LCMS and the peptide was purified by HPLC (*Figure 4-5*).

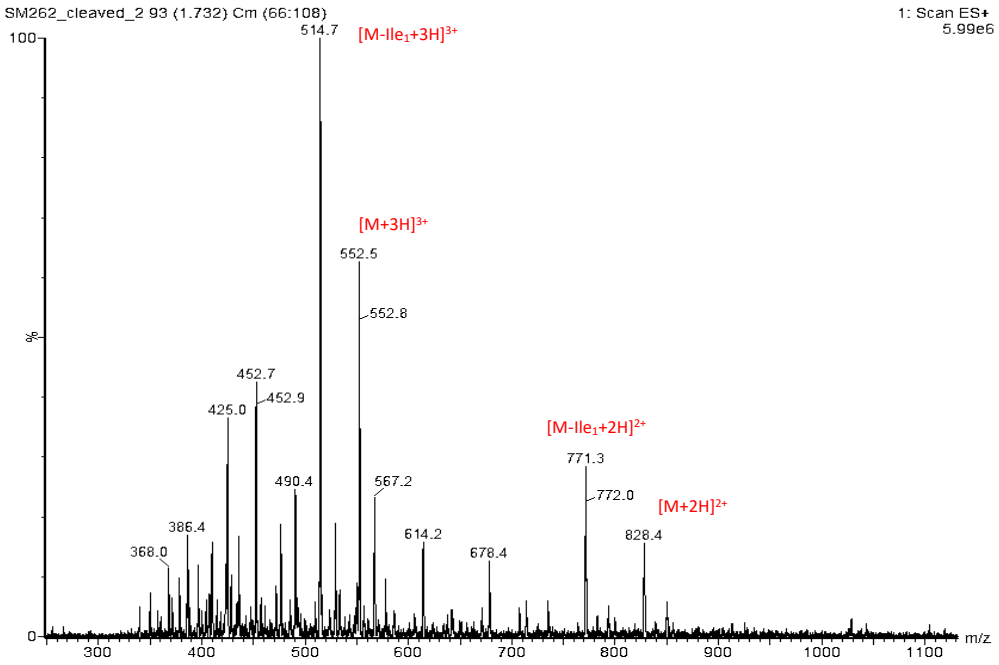


*Figure 4-4: Crude mass spectrum of mastoparan (**12**) synthesis without double coupling*

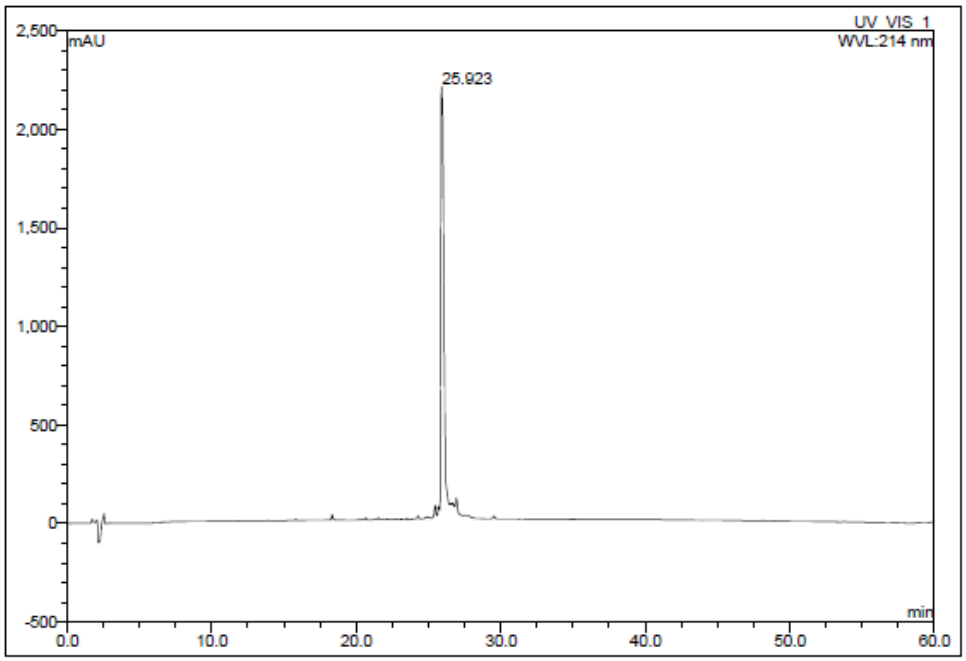


*Figure 4-5: Analytical HPLC chromatogram of purified matoparan (**12**) obtained using the standard conditions as described in Chapter 7.3*

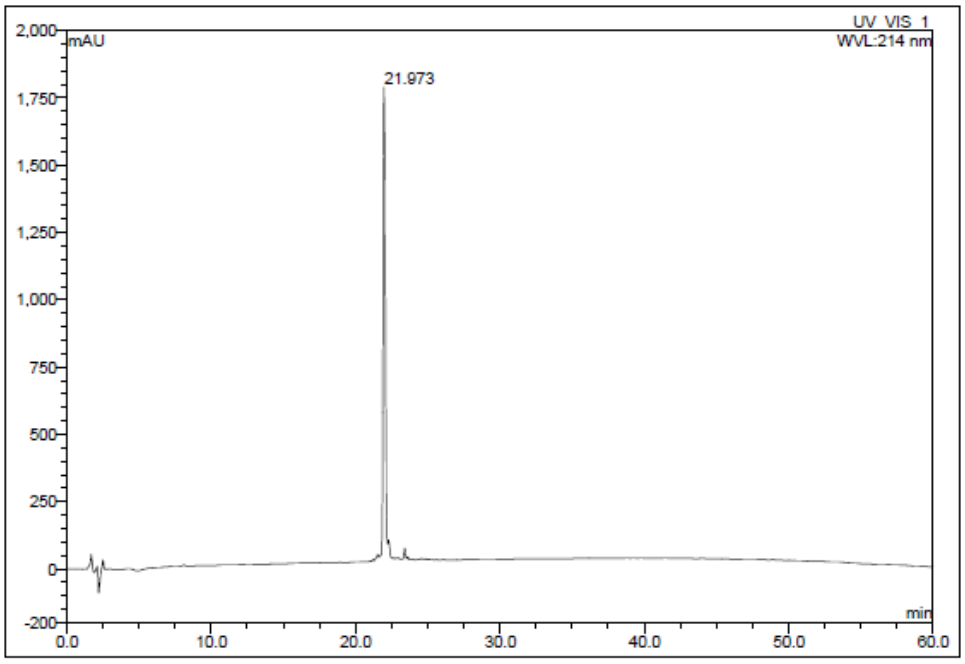
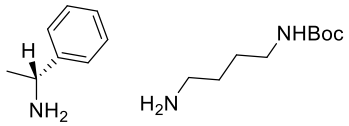


*Figure 4-6: Analytical HPLC chromatogram of purified pexiganan (**10**) obtained using the standard conditions as described in Chapter 7.3*

4.2.2 Peptoid Synthesis

Peptoid synthesis was carried out using the procedure previously reported by Barron and Patch using the amine monomers (*S*)-(-)-α-methylbenzylamine (MBA) (**169**) and mono-Boc

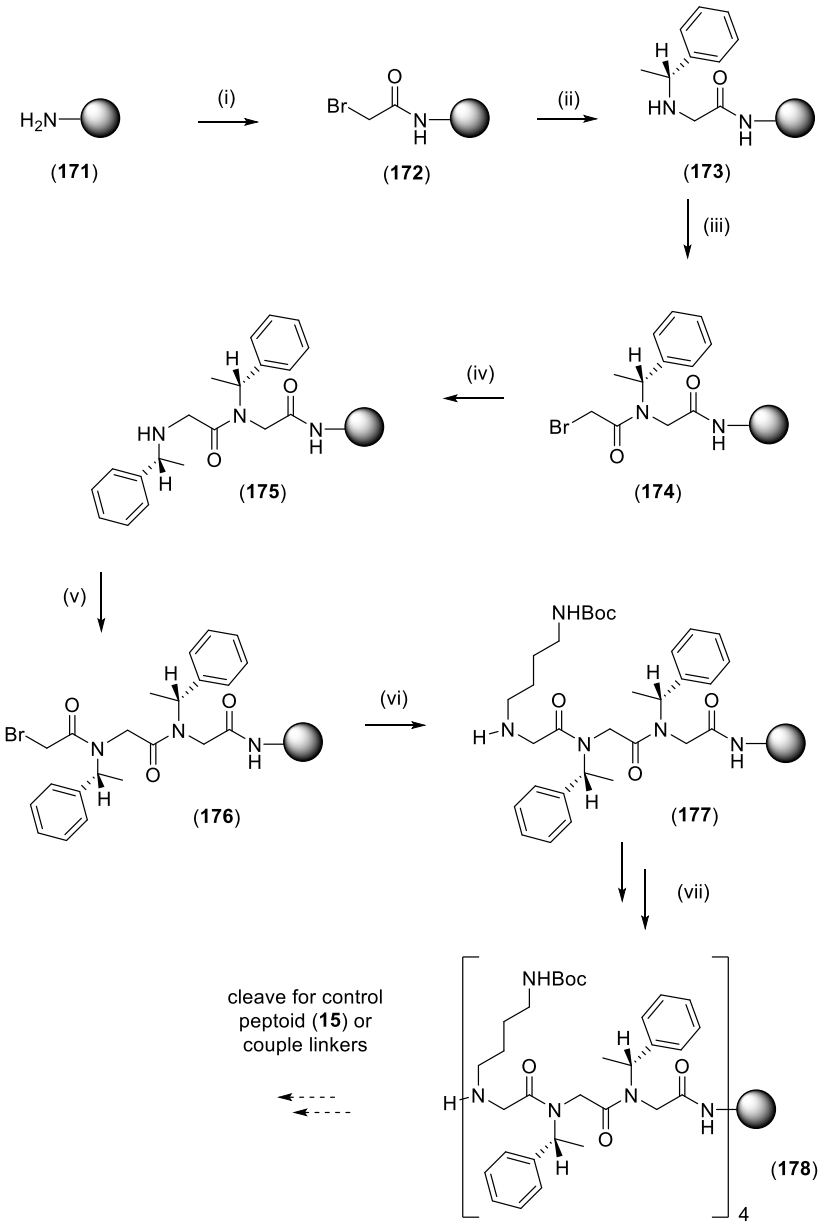
protected dibutamine (**170**).[85] The synthesis of Boc protected dibutamine (**170**) was attempted but the starting material diamine was toxic and difficult to handle on a medium scale and so (**170**) was instead purchased from Sigma Aldrich.



(**169**) (**170**)

Figure 4-7: Amine monomers used in peptoid synthesis

Rink amide resin was again used to give the C-terminal amide. The automated peptide synthesiser was programmed to carry out addition of bromoacetic acid in DMF to the resin and then addition of DIC in DMF to activate the acid. After 1 h this was followed by washings with DMF and NMP and then subsequent additions of the required amine followed by washings of NMP and DMF (*Scheme 4-1*).



Scheme 4-1: Peptoid synthesis

*Reagents and conditions: (i) bromoacetic acid and DIC added to the reaction syringe, after 1 h syringe was evacuated and washed with 3 portions of DMF followed by 3 portions of NMP (ii) (**169**) in NMP added to the reaction syringe and after 1 h the syringe was evacuated and washed with 3 portions of NMP followed by 3 portions of DMF (iii) repeat of bromoacetic acid coupling (iv) repeat of amine coupling with (**169**) (v) repeat of bromoacetic coupling (vi) (**170**) in NMP added to the reaction syringe and after 1 h syringe was evacuated followed by washing with 3 portions of NMP followed by 3 portions of NMP (vii) repeat of steps (i-vi) x 4*

The synthesis was successful showing adequate purity in the crude LCMS and peptoid (**15**) was purified by reverse phase HPLC in adequate yield (5%). Due to the lack of backbone chirality peptoids exhibit greater flexibility and the increase in conformational freedom results in broadened peaks on HPLC (*Figure 4-8*).[83]

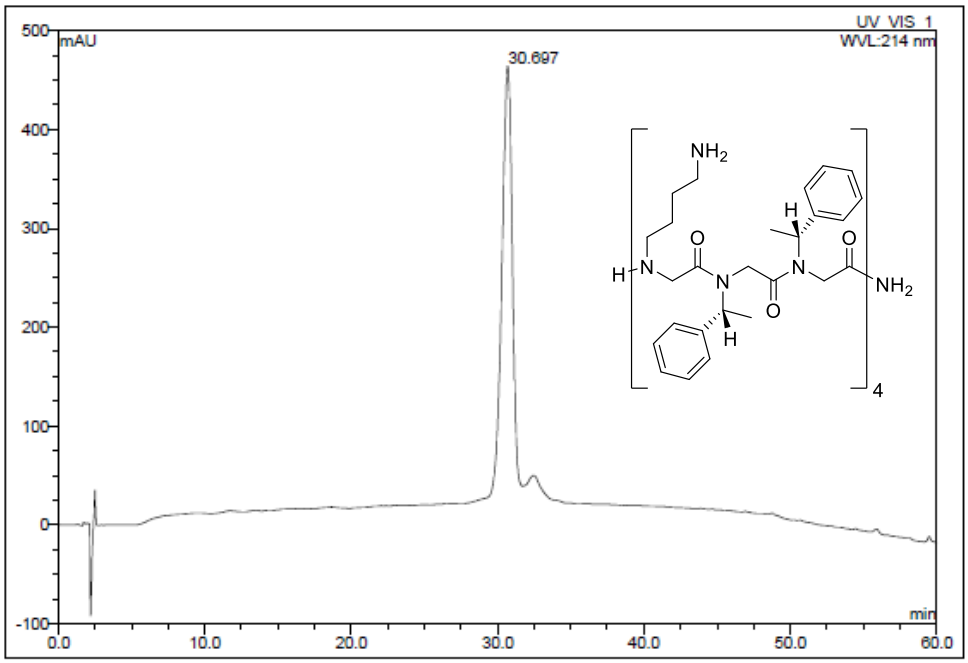
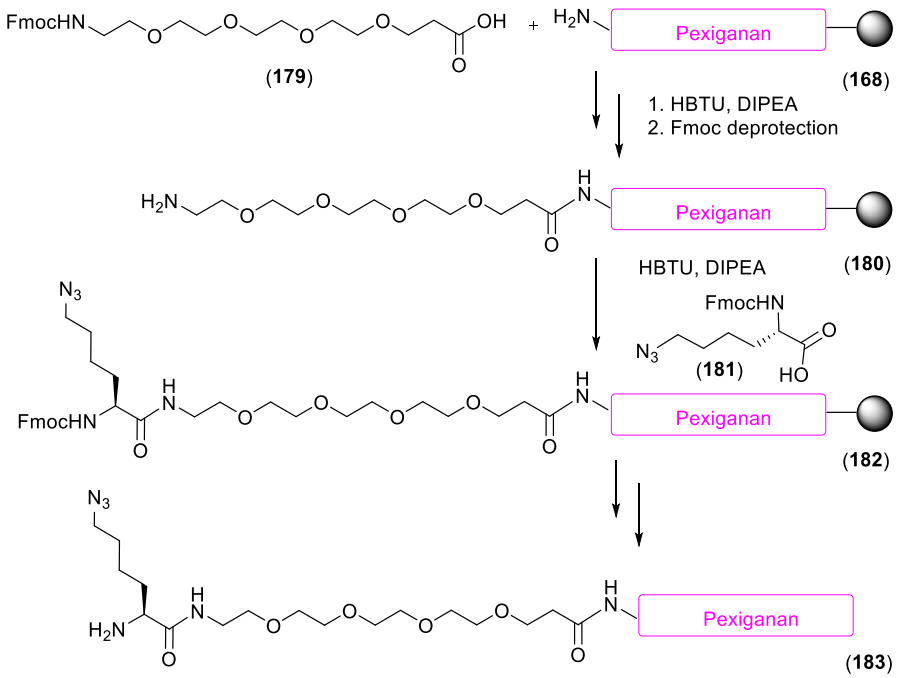


*Figure 4-8: Analytical HPLC chromatogram showing broadened peak of purified peptoid (**15**) using the standard conditions as described in Chapter 7.3*

4.3 Hinge Region Linkers

The first set of linkers were chosen from commercially available reagents available within the laboratory. As previously mentioned PEG linkers are extremely suitable for this role and so Fmoc protected PEG4 amino acid (**179**) and Fmoc-Lys(N_3)-OH (**181**) were employed as this was a straightforward way to incorporate an azide moiety. These were coupled using standard amide coupling conditions directly onto the N-terminus of resin bound intermediates of mastoparan (**167**) and pexiganan (**168**) before being cleaved from resin and purified (*Scheme 4-2*).

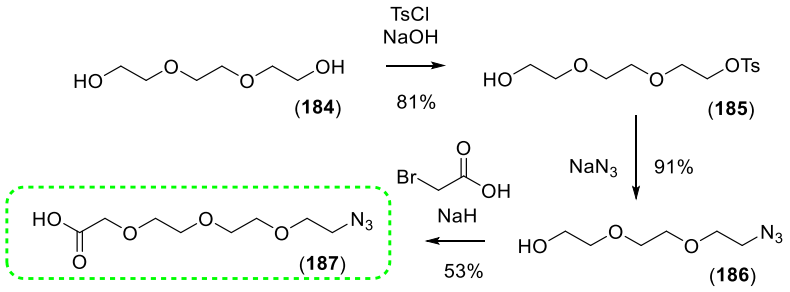


Scheme 4-2: Incorporation of PEG4 and Fmoc-Lys(N_3) onto N-terminus of pexiganan

Hinge region mimics produced in this way were significantly longer than the nisin hinge region and so shorter linkers were then designed.

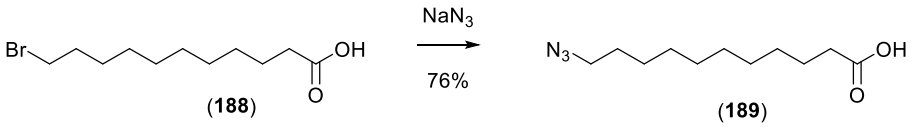
Shorter PEG linker, 11-azido-3,6,9-trioxaundecanoic acid (**187**) was chosen as the next target linker. This PEG linker, and longer chain analogues, have been synthesised from triethylene glycol, or longer PEG compounds, by a number of groups often as an intermediate in the synthesis of PEG amino acids where the azide group is later reduced to an amine.[222] The azide is commonly installed by substitution of a mono-mesylated PEG chain with sodium azide.[222,223] The acid can be installed in a number of ways such as oxidation of the terminal alcohol[224] or installed as an ester and later hydrolysed.[223]

Here the route of Weong and O'Brien[225] was used which installed the acid using a substitution reaction with bromoacetic acid (*Scheme 4-3*). Synthesis began with mono-tosylation of triethylene glycol (**184**) and then substitution with sodium azide to give (**186**). Initially the conditions of Jeong[225] were used to install the acid moiety, using potassium hydroxide as a base, but this was unsuccessful and despite several attempts, the product (**187**) was never obtained with >5% yield. The conditions of Behrens *et al.*[226] were then attempted which used sodium hydride as base and this was successful giving (**187**) in 53% yield.



*Scheme 4-3: Synthesis of PEG3 linker (**170**)*

Inspired by the good lipid II binding activity of the lipopeptides synthesised by Koopmans *et al*.[98] an alkyl hinge region (**189**) was designed to investigate whether strong binding to lipid II could be combined with the pore forming ability of one the tail peptides. However, it would be expected that the incorporation of the alkyl chain may decrease aqueous solubility.



*Scheme 4-4: Synthesis of alkyl linker (**172**)*

The alkyl linker (**189**) was easily synthesised from commercially available 11-bromoundecanoic acid (**188**) by refluxing with sodium azide to obtain (**189**) in good yield.

4.4 Target Tail + Linkers

With the linkers in hand, a set of tail peptides coupled with linkers was synthesised (*Figure 4-9*). Linkers were coupled onto the N-terminus of resin bound intermediates (**167**), (**168**) and (**178**) using 5 equivalents of linker and standard HBTU and DIPEA coupling conditions. Double coupling was used to ensure the reaction went to completion. The tail peptides plus linkers were then cleaved from resin and purified to give multi milligram amounts in good yield. These tail peptides were now ready to undergo click chemistry.

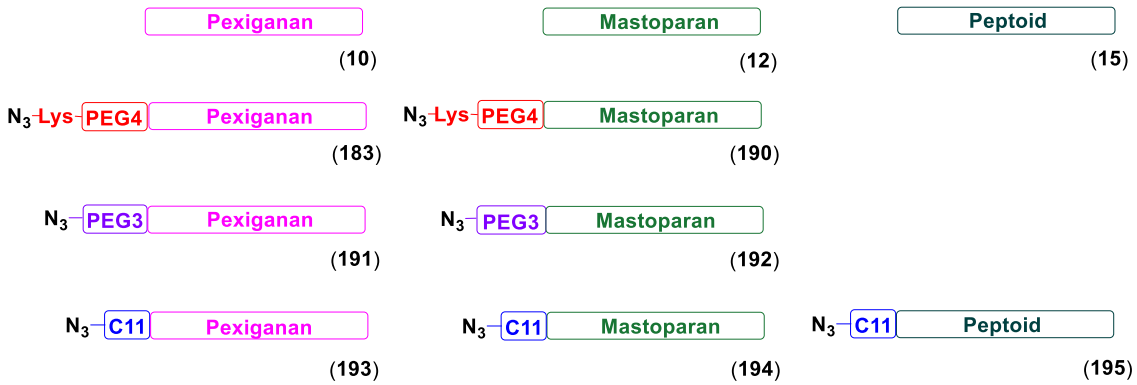
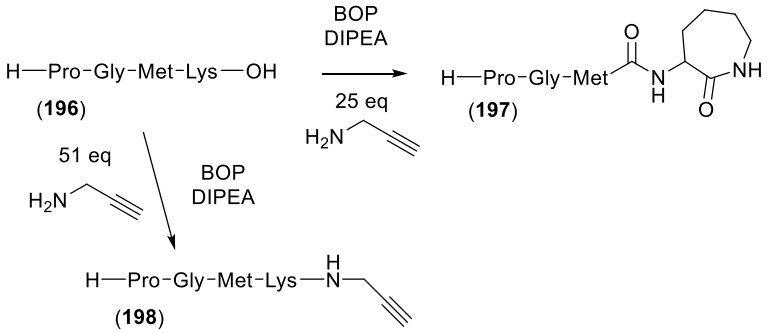


Figure 4-9: Set of target tails coupled with each linker; the full set of peptoid tails were not synthesised due to time constraints

4.5 Model Click Chemistry

Model click reactions were carried out to optimise the conditions required for click chemistry, minimising the loss of valuable intermediates already synthesised. To this end, model peptides were synthesised to represent the C-terminus of rings AB (**196**) and the N-terminus of a tail peptide, pexiganan (**199**), terminated with Fmoc-Lys(N_3)-OH (**181**). The first step was to check the conditions used for coupling an alkyne moiety onto the model rings AB peptide (1**96**). This was carried out using the conditions of Slootweg and co-workers who coupled propargylamine onto the C-terminus of full length WT nisin using 25 equivalents of propargylamine (*Scheme 4-5*).[179]

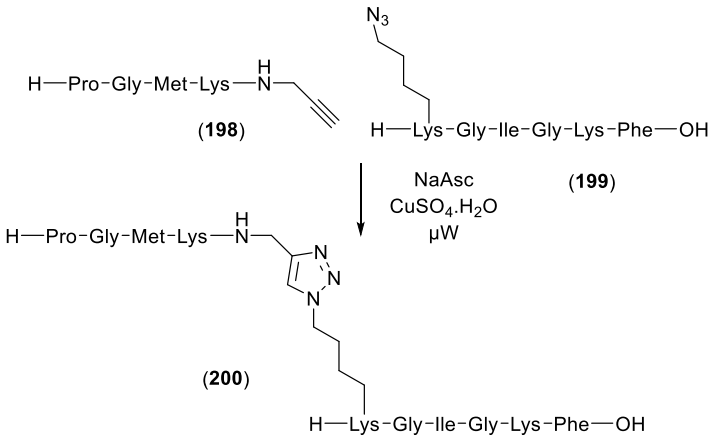


*Scheme 4-5: Coupling of propargylamine to ring B N-terminus model (**198**)*

Using these conditions, no visible peak for the alkyne product (**198**) could be observed in the crude LCMS. A peak of *m/z* = 414.4 was observed which was attributed to peptide (**197**), produced by the intermolecular cyclisation of the side chain amine of lysine coupling with itself. Other researchers who had coupled an amine handle to rings AB of nisin had

also observed the intramolecular cyclisation of the terminal lysine of rings AB.[98] They suggested that more than 50 equivalents of amine were required to avoid the cyclisation and so the reaction was repeated using 51 equivalents of propargylamine and this gave alkyne-peptide (**198**) in good yield, 55%.

With the truncated AB-alkyne and pexiganan mimics in hand, click chemistry was attempted using 0.2 equivalents of $CuSO_4.H_2O$ and 0.4 equivalents of NaAsc, heating in the microwave for 15 min at 80 °C using the smallest microwave vial available at the time, 5 mL, to give a concentration of 1.65 mM (*Scheme 4-6*). Product (**200**) was observed in the crude LCMS alongside peaks for both starting materials suggesting the reaction had not gone to completion. An extra 0.2 equivalents of $CuSO_4.H_2O$ and 0.4 equivalents of NaAsc was added to the reaction vial and the vial was returned to the microwave for a further 15 min at 80 °C. The reaction did appear to have progressed further but starting materials were still visible. This prompted a short review of literature conditions for click chemistry involving fragments of nisin.



Scheme 4-6: Model click chemistry

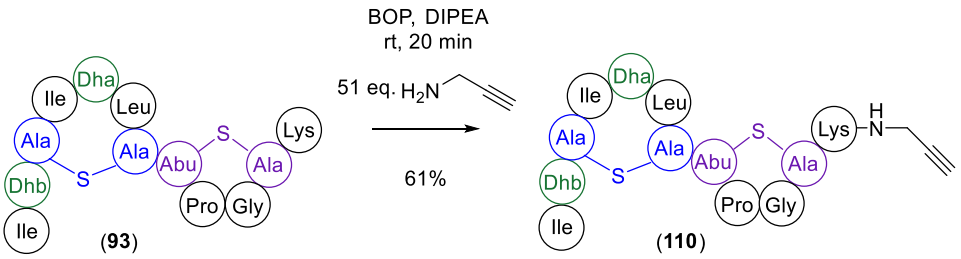
Reference	Reagents	Time (mins)	Temperature (°C)	Concentration (mM)	Yields (%)
Slootweg *et al.*[183]	1.9 eq $CuSO_4.H_2O$ 5.95 eq NaAsc	20	80	0.737	26-54
Koopmans *et al.*[98]	0.2 eq $CuSO_4.H_2O$ 0.4 eq NaAsc 0.05 eq TBTA	20	80	16.2	13-60
Arnusch *et al.*[184]	2.0 eq $CuSO_4.H_2O$ 5.0 NaAsc	5	80	1.90	39
Arnusch *et al.*[186]	5.1 eq $CuSO_4.H_2O$ 10 eq NaAsc	10	80	1.43	53-79

Table 4-1: Review of literature conditions for click chemistry involving fragments of nisin

Click chemistry was then attempted with increased equivalents of NaAsc and $CuSO_4$, (5.1 and 10) using the conditions of Arnusch *et al.*[184] with a smaller microwave vial, 2 mL. Peaks for starting materials were still visible in the crude LCMS although at a decreased intensity. Concentrating the reaction down further by using a 0.5 mL vial, to give a concentration of 15.7 mM, pushed the reaction to completion with no starting materials visible in the crude LCMS. This reaction was repeated following the procedure of Koopmans *et al.*[98] with the addition of tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) but no visible difference could be seen in the yield of product (**200**) as assessed by HPLC.

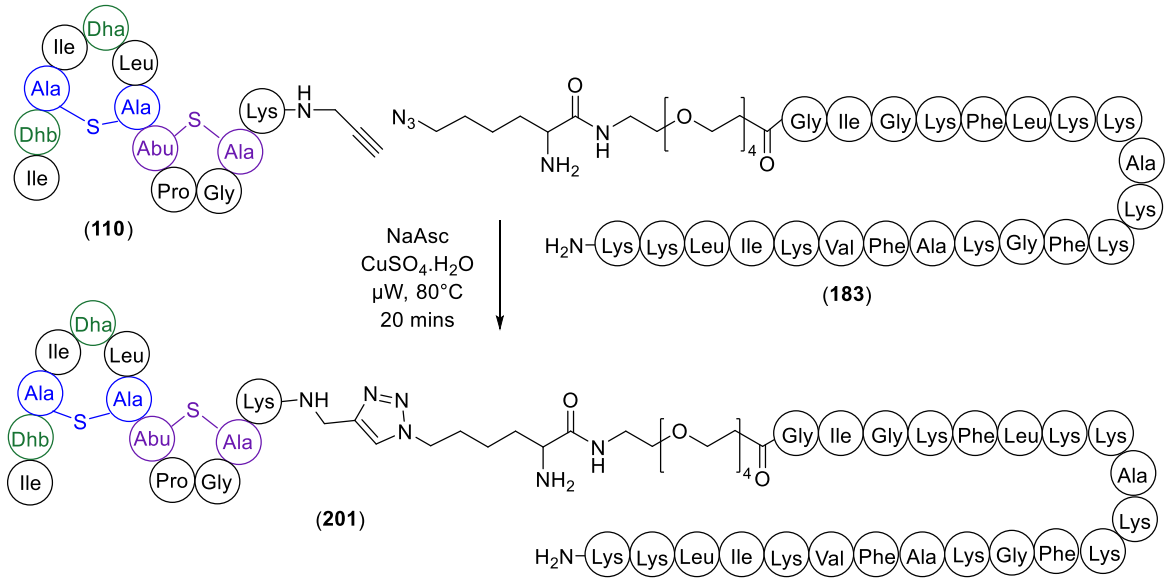
4.6 Click Chemistry

Using the optimised conditions from the model experiment, propargylamine was coupled to rings AB in adequate yield (*Scheme 4-7*).



Scheme 4-7: Coupling propargylamine to rings AB

Click chemistry was then performed with each of the tail peptides. The first click reaction was attempted using (N_3)Lys-PEG4-pexiganan (**183**) and the optimised conditions of the model click reactions without TBTA. This was successful and gave (**201**) with a yield of 8%. The second click reaction was attempted with N_3-C11-mastoparan (**194**) using the same conditions. This was also shown to be successful to give (**205**, *Figure 4-11*) with a yield of 8%.



Scheme 4-8: Synthesis of the first hybrid peptide

The third click reaction with N_3-PEG3-pexiganan (**191**) was unsuccessful with only starting materials collected. The fourth with (N_3)Lys-PEG4-mastoparan (**190**) also failed. After two failed click reactions it was decided that TBTA[227] would be used as a catalyst, as used by Koopmans *et al.*,[98] and so all further click reactions were carried out with 0.05 equivalents of TBTA (**202**).

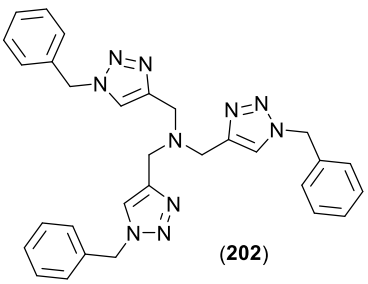


*Figure 4-10: Structure of ligand TBTA (**202**)*

TBTA is a polytriazole ligand designed as a stabilizing ligand for copper(I) protecting it from oxidation and disproportionation and so improving catalytic activity.[227] Cu(II) ions have been shown to catalyse the oxidative coupling of alkyne substrates to give diynes as undesired by-products. Fokin and co-workers, the researchers who designed TBTA, proposed that TBTA acts as a tetradentate ligand and blocks all coordination sites at the metal centre so that no oxidant can attack at the copper(I) ion.[227] The tertiary amine was assumed to bind permanently to the metal centre, donating additional electron density to the metal centre whilst the triazole ligands would temporarily dissociate from the copper(I) ion to allow for the formation of the copper acetylide complex.

Here it was employed with success, the reaction of N_3Lys-PEG4-mastoparan (**190**) with alkylated rings AB was repeated with TBTA and the product (**203**) was obtained with 5% yield. Five further click reactions were carried out successfully with only one further failure with N_3-C11-pexiganan (**193**). This reaction was not repeated due to time constraints.

Substrate	TBTA	Product observed
(N_3)Lys-PEG4-pexiganan (183)	×	✓
N_3-C11-mastoparan (194)	×	✓
N_3-PEG3-pexiganan (191)	×	×
N_3Lys-PEG4-mastoparan (190)	×	×
N_3-PEG3-mastoparan (192)	✓	✓
N_3Lys-PEG4-mastoparan (190)	✓	✓
N_3-C11-pexiganan (193)	✓	×
N_3-C11-peptoid (195)	✓	✓
(N_3)Lys-PEG4-pexiganan (183)	✓	✓
N_3-C11-mastoparan (194)	✓	✓

Table 4-2: Summary of click reactions

The first full set of hybrid peptides obtained were the mastoparan analogues (**203**-**205**, *Figure 4-11*) and these underwent initial assessment for antimicrobial activity as discussed in Chapter 5.

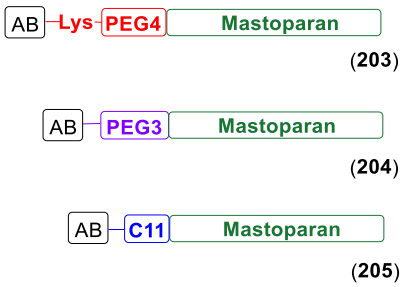


Figure 4-11: Mastoparan hybrids taken on for biological evaluation

Initial biological testing was promising with all three peptides showing antibacterial activity against *Micrococcus luteus*, an "indicator strain" for activity in Gram positive bacteria. Unfortunately, time constraints meant that only four hybrid peptides were obtained in high enough quantity to be taken on for full biological evaluation and determination of MICs (*Figure 4-12*).

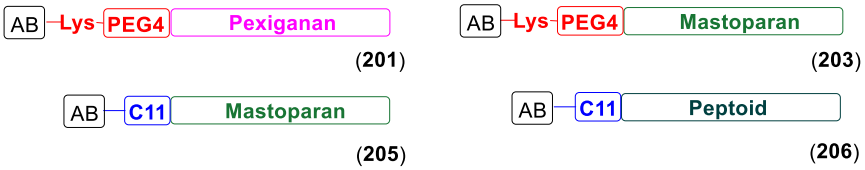


Figure 4-12: Hybrid peptides taken on for MIC determination

4.7 Summary

The synthesis of mastoparan (**12**), pexiganan (**10**) and peptoid (**15**) were carried out according to literature procedures. Hinge region linkers were then synthesised and coupled to each of the tail peptides to generate a novel set of tail peptides + linkers. Concurrently, propargylamine was coupled to WT nisin rings AB (**110**). Click chemistry methodology was developed using model peptides and this work enabled the synthesis of six novel hybrid peptides (**201**, **202**-**206**) which are assessed for biological activity in Chapter 5. Low yields for this work can be attributed to the difficulties of handling and purifying small scale synthesis as the final click reactions were shown to proceed cleanly to completion as visible by HPLC.

5 *In vitro* Biological Testing

As the pressure to produce new antimicrobials increases, so does the need for better screening methods that quantify biological activity, particularly, *in vitro* methods that provide accurate predictions for *in vivo* activity. Two of the most commonly used methods within the field of AMPs are agar well diffusion and broth dilution methods both of which will be discussed in this chapter. For a recent review on the full range of methods see Balouiri e*t al*.[228] Good agreement between the minimum inhibitory concentrations (MICs) obtained from using the different methods has been observed[229,230] and a handful of different regulatory protocols exist in order to standardise MIC determination as much as possible, for example, the Clinical and Laboratory Standards Institute (CLSI) in the USA and the European Committee on Antimicrobial Susceptibility Testing (EUCAST)[231] in Europe.

Agar well diffusion methods use nutrient agar plates innoculated with a defined number of bacteria that are spread over the entire agar surface.[228] Wells are then punched into the agar and solutions of the antibiotic are spotted into the cavities prior to incubation. Alternatively, absorbent discs can be pre-soaked in an antibiotic solution and placed on top of the agar, alleviating the need for holes. Decreasing concentrations of antimicrobial are spotted into the wells or onto the discs and the MIC is determined when a “halo” of growth inhibition can no longer be observed. The size of the inhibition zone is attributed to the antimicrobial activity of the compound tested and the diameter can be used as a quantitative measurement of activity.

For the broth dilution method, a liquid growth medium is used and the amount used can be varied depending on the scale required and the availability of equipment.[228] On a larger scale, macro-dilution, around 2 mL of broth can be used and this can be carried out in falcon tubes. For a smaller scale, micro-dilution, up to 500 µL of broth can be used and this is usually carried out in microtitre plates. Typically, a range of concentrations of antimicrobial compound are generated by carrying out a two-fold serial dilution. The MIC is determined by the lowest concentration at which no turbidity or sediment (indicating bacterial growth) can be visibly observed. The optical density can also be measured as a quantitative measurement of bacterial growth.

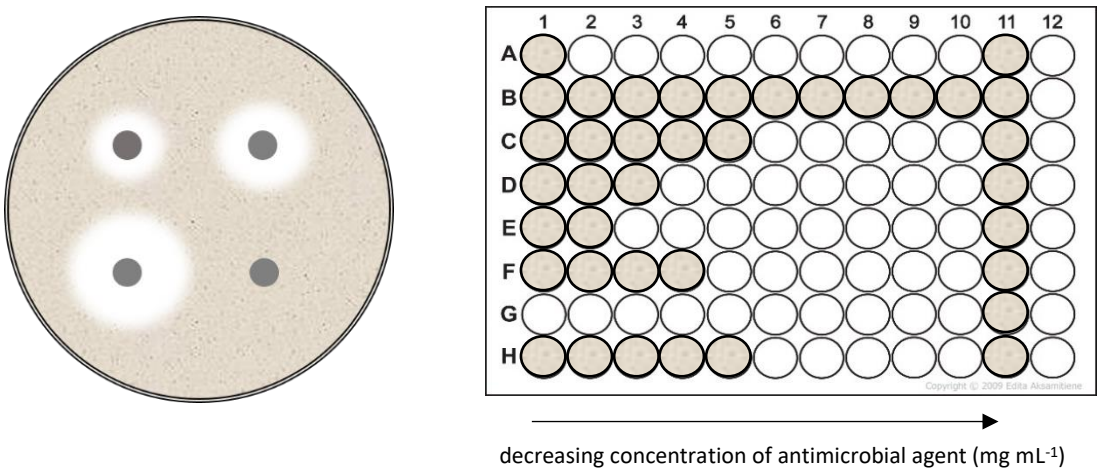


Figure 5-1: Diagram of an agar plate with four wells (dark grey) and varying sizes of inhibition zones (white) (left); and a typical set up of a broth micro-dilution in a 96 well plate (right); each row can contain a different antimicrobial agent or a different strain of bacteria; shading indicates bacterial growth, for example, row B shows no inhibition of growth; column 11: growth control, column 12: sterility control

Neither of these techniques provide information on the nature of inhibition - whether the antimicrobial is bactericidal (kills bacteria) or bacteriostatic (prevents growth) - and this is usually determined by further assays to produce a time-kill curve.

5.1 Initial Testing - Agar Well Diffusion

Assay work was performed by S. Mitchell and stock solutions of bacteria were prepared by Prof J. Ward.

For the initial round of testing, the agar well diffusion method was used as this was simple to set up and a protocol was already in use within the laboratory. Four different strains of bacteria were tested. Two indicator strains of Gram positive bacteria, *Micrococcus luteus* and *Bacillus subtilis* and two strains of Gram negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*. The first set of hybrids synthesised were the mastoparan analogues (**203**-**205**, *Figure 5-2*) and so these were used in initial testing. A number of controls were also used: Kanamycin, an antibiotic in clinical use; mastoparan (**12**); WT rings AB (**93**); full length WT nisin (**27**).

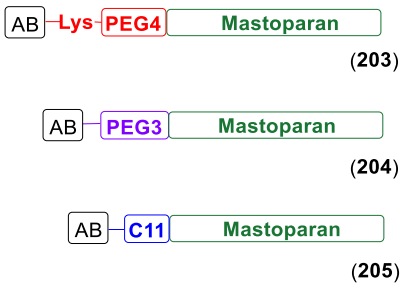


Figure 5-2: Hybrid peptides tested using agar well diffusion

All of the peptides and peptoids synthesised were cleaved in TFA and purified using a solvent system with 0.1% TFA and so it was assumed that all of the cationic residues exist as TFA salts. Due to the high number of cationic residues, the TFA content makes up a significant proportion of the isolated mass. It has previously been reported that the presence of TFA can inhibit bacterial growth in assays[232] so to check if it would have an effect in the assays, a control solution of sodium trifluoroacetate was tested for antibacterial activity. It was made up to the same concentration as the sample with the highest concentration of TFA salt. No inhibitory activity was observed in agar well diffusion plates at the maximum amount tested, 25 µL of 0.4 mg mL^{-1}.

It was assumed that the hybrid peptides would have better activity against Gram positive bacteria, however, as mentioned in Chapter 1 the tail peptides have been shown to exhibit some antimicrobial activity against Gram negative bacteria. The mastoparan and pexiganan analogues were reported to have the most promising activity showing only a two-fold and four-fold increase in MIC against *E. coli* compared to *B. subtilis* respectively.[21,86]

All peptides were made up to 1 mg mL^{-1} stock solutions in sterile water. An overnight culture of each bacteria strain was prepared by Prof. J. Ward and 100 µL of each were pipetted and spread onto plates of L-Agar. Four holes were punched into each plate and 25 µL of peptide stock solution was spotted in each well. The plates were incubated for 48 hours at 37 °C except for *M. luteus* samples which were incubated at 30 °C.

5.2 Results

Inhibition zones were visible for all hybrid peptides and controls against *M. luteus*. Of the hybrids only AB-PEG3-mastoparan (**204**) showed slight inhibition of *B. subtilis*, with a small zone of inhibition, but the controls nisin, mastoparan and Kanamycin all showed good

inhibition. Rings AB only showed inhibition against *M. luteus*. As expected, all peptides performed worse against the Gram negative bacteria with only Kanamycin showing any inhibition against the Gram negative bacteria (*Table 5-2*).

Peptide	Activity against *M. luteus*	Activity against *B. subtillus*	Activity against *E. coli*	Activity against *P. aeruginos*
AB-Lys-PEG4-Mastoparan (203)	✓	×	×	×
AB-PEG3-Mastoparan (204)	✓	✓	×	×
AB-C11-Mastoparan (205)	✓	×	×	×
Mastoparan (12)	✓	✓	×	×
Nisin AB (93)	✓	×	×	×
Nisin (27)	✓	✓	×	×
Kanamycin	✓	✓	✓	✓

Table 5-1: Activity of hybrid peptides and controls determined using agar well diffusion
✓: zone of inhibition visible, ×: no zone of inhibition visible
Plates spotted with 25 µL of peptide and Kanamycin stock solutions made up to 1 mg mL^{-1}

5.3 MIC Determination - Broth Micro-dilution

Assay work was performed by S. Mitchell. Preparation of stock solutions of bacteria and colony counts were performed by Prof J. Ward

To determine the MIC of the hybrid peptides and to compare their activities more accurately the broth micro-dilution method was used as this consumed the minimum amount of peptide material. Due to limited amounts of peptides, further testing was also only carried out on *M. luteus* as this appeared to be the most sensitive strain of Gram positive bacteria.

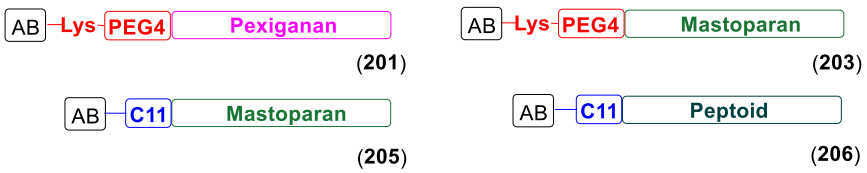


Figure 5-3: Hybrid peptides that were taken on for MIC determination

The assays were carried out in 96-well plates using the broth micro-dilution method outlined by Hancock and co-workers.[233] The MIC of each peptide was defined as the lowest

concentration of the assayed peptide that inhibits the visible growth of the microorganism tested. The optical density of the plates was also recorded.

Polystyrene 96-well plates have been reported to inhibit the activity of cationic AMPs as it is believed the peptides bind to the negatively charged surface of the plastic.[233] Polypropylene plates were used for the assay to mitigate this effect. To aid solubility, 1% DMSO final volume was used throughout.

The initial biological testing using agar well diffusion compared the peptides as a weight per volume concentrations but due to the differences in molecular weight and assumed TFA salt content it was decided that using a mole per volume concentration scale would give a clearer indication of the differences in activity. Controls of nisin (**27**), mastoparan (**12**), pexiganan (**10**), peptoid (**15**) and rings AB of nisin (**93**) were tested alongside the hybrids.

All assays were performed in duplicate. 10 µL of an overnight stock of bacteria, as prepared by Prof. J. Ward, was used in each well. Colony counts performed by Prof. J. Ward on the overnight stock culture of bacteria gave a count of 1.07×10^7 cfu mL^{-1}, however, the final concentration in each well is diluted by a factor of 25 and so the initial concentration of bacteria in each well was estimated at 4.28×10^5 cfu mL^{-1}. Column 11 of the plates was used as a control for bacterial growth and this column contained only 10 µL of bacteria stock solution and 240 µL of broth. Column 12 of the plates was used as a control for sterility and contained 250 µL of broth. The plates were incubated for 20 hours at 37 °C with shaking after which the broth with any bacterial growth from each well was re-suspended and transferred to a transparent polystyrene plate to measure the optical density.

Initial broth-microdilution assays using the control peptides suggested the MICs would fall below 12 µM and so this was used as the initial concentration from which a two-fold serial dilution was carried out using the method suggested by Hancock and co-workers.[233] Initial assays also suggested that the choice of growth medium was important. LB broth was initially used as growth medium for the overnight stocks of bacteria and this gave good growth of bacteria as indicated by a cloudy solution. However, when LB broth was used in the broth-microdilution assays, poor growth of the bacteria was visible with an optical density of around 0.2 for the growth control column and wells where growth inhibition was not expected. Oxoid Nutrient Broth No 2 was instead used for overnight culture medium and as medium in the broth-microdilution assays. This gave good bacterial growth was seen

with an optical density around 0.4 in the growth control column and wells where growth inhibition was not expected. Oxoid Nutrient Broth No 2 with 1% DMSO was then used for all overnight growth cultures and micro-dilution assays.

5.4 Results

As expected, nisin (**27**) showed greater activity than any of the hybrids or linear peptides. All but one of the hybrids (**205**) were shown to have greater activity than rings AB suggesting that pore forming activity was restored to the lipid II binding motif *(Figure 5-4*). However, as none of the hybrids performed better than their tail component controls it is not conclusive that their activity is conferred by a lipid II dependant mechanism. It could be that the linear peptides are behaving as they would do in isolation and the AB rings are not disturbing that activity significantly. Further experiments using lipid II spiked LUVs, such as those used by Breukink and co-workers,[186] are required to probe the mechanism.

Notably, the alkyl, C11 linked hybrids (**205**) and (**206**) exhibited the worst activity compared with the PEG4-lysine linked hybrids (**201**) and (**203**). The best of which (**201**) matched the activity of its tail peptide, pexiganan (**10**). The poorer activities may be because the alkyl linker disrupts the ability of the mastoparan and the peptoid tails to form a helix or possibly due to reduced solubility. The C11 linked hybrids also have the shortest tails of 14 residues and 12 monomers respectively. In comparison, the best performing hybrid (**201**) which has the longest peptide tail of 22 residues. It may be that the longer peptide tails can span the bacterial membrane better and form more effective pores. The hybrid (**201**) also has the highest number of cationic residues and is the least hydrophobic compared to the other hybrids as estimated by HPLC retention time however there is not enough evidence available to establish trends within these parameters.

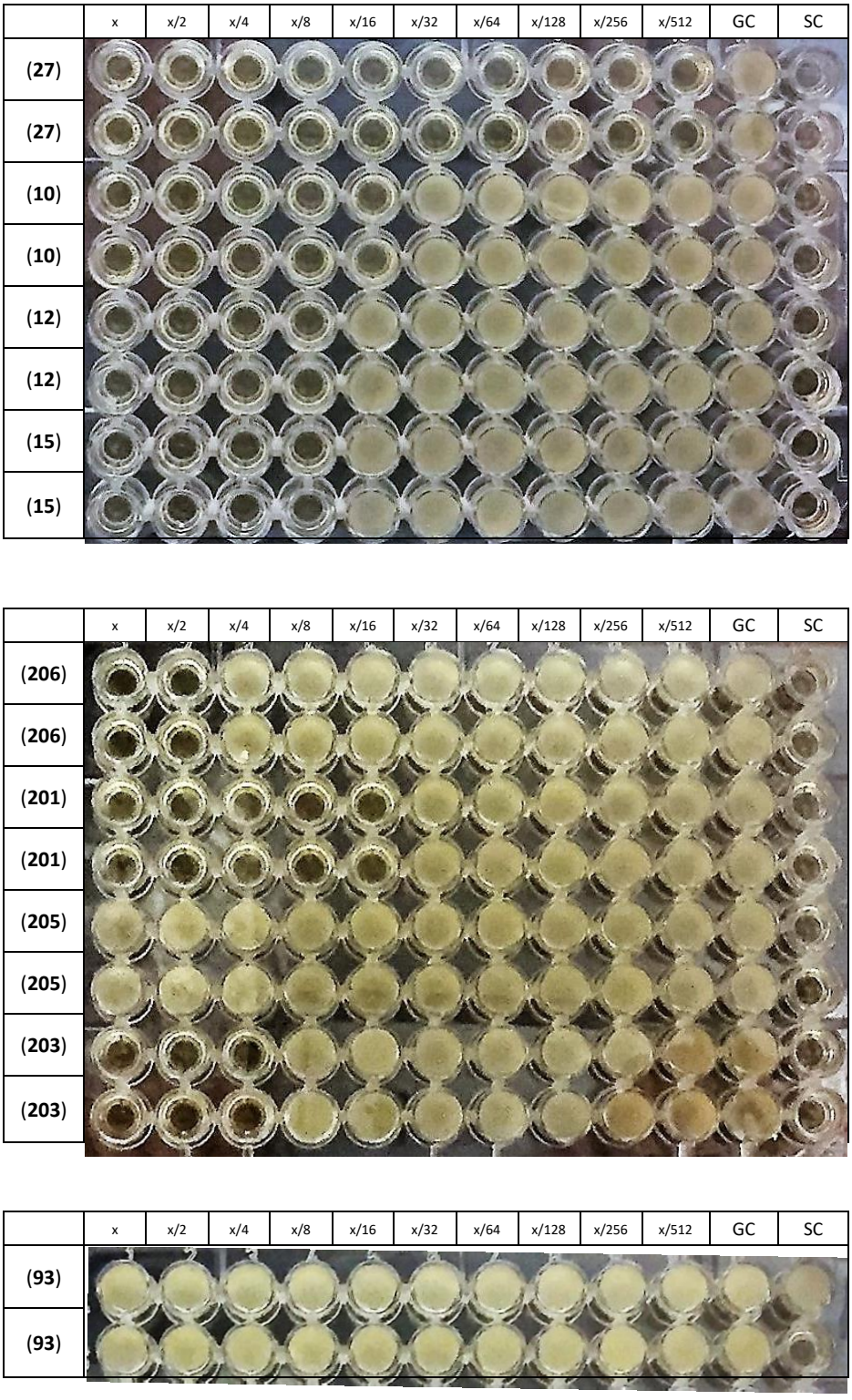


Figure 5-4: Photographs of microdilution assay; each row contains decreasing concentration of peptide left to right; x = 12 µM; clear wells indicate bacterial growth inhibition, cloudy wells indicate bacterial growth

Peptide	MIC (μM)	No. of Cationic Residues	HPLC Retention Time (mins)
Nisin (27)	0.0234	5	28.0
Pexiganan (10)	0.750	9	22.9
Mastoparan (12)	1.50	3	25.9
Peptoid (15)	1.50	4	30.7
AB (93)	>12.0	1	28.2
AB-Lys-PEG4-Pexiganan (201)	0.750	11	24.9
AB-Lys-PEG4-Mastoparan (203)	3.00	5	26.2
AB-C11-Mastoparan (205)	>12.0	4	31.0
AB-C11-Peptoid (206)	6.00	5	32.8

Table 5-2: MICs of hybrid peptides against M. luteus determined by broth micro-dilution assays; number of cationic residues or monomers (Arg, His, Lys or NLys) shown; HPLC retention time acquired using standard conditions as described in Chapter 7.3

Although the MIC of AB-C11-mastoparan (**204**) did not show visible inhibition of *M. luteus*, when the optical density was taken the highest concentration of peptide does appear to show some decrease in optical density and it could be assumed that the MIC will fall within a fourfold increase in concentration (*Figure 5-5*).

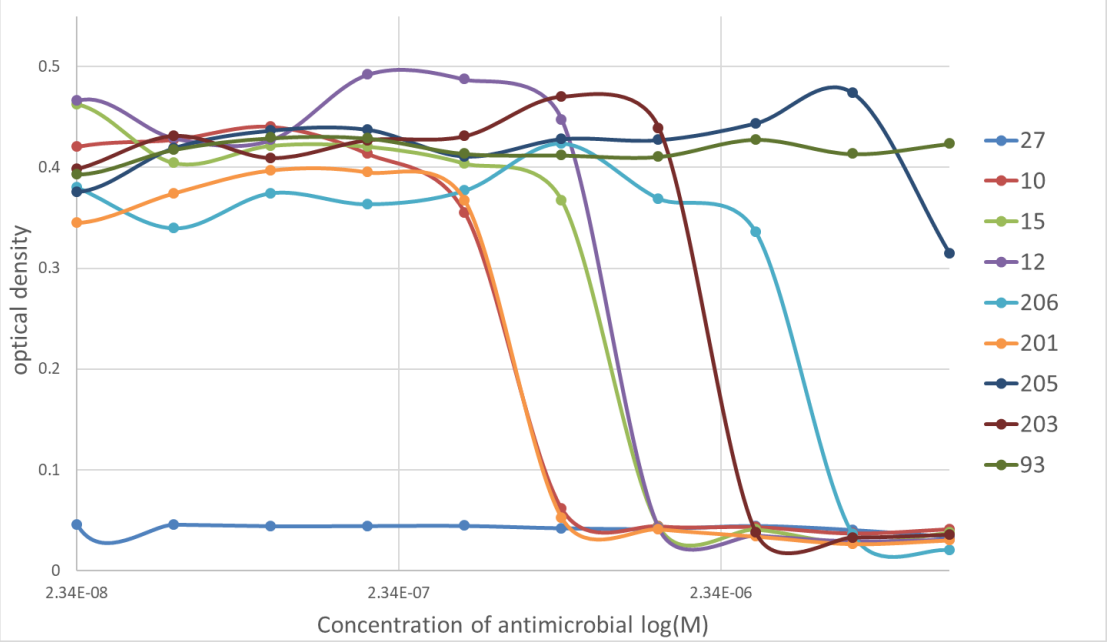


Figure 5-5: Graph showing optical density vs concentration of peptide of broth micro-dilution assays

5.5 Summary of *in vitro* Testing

Three out of the four hybrids (**201**, **203**, **206**) were shown to have good activity against *M. luteus,* in the low μM range, likely due to their membrane destabilising, pore forming ability. It was inconclusive whether this occurred through a lipid II binding mechanism and further testing needs to be carried out to establish this.

The next stage of assessing the biological activity of the hybrid peptides should also involve assessment of activity against more clinically relevant strains of bacteria for example against MRSA and VRE strains.

6 Conclusions and Future Work

The aim of this project was to prepare simplified mimics of nisin with improved drug-like properties and an accessible synthetic route. To preserve the selectivity and activity of nisin it was necessary to design mimics with conserved AB ring moieties. By extending SPPS methodology previously developed within the group, the AB ring moiety of nisin was simplified by replacing unstable dehydro residues and substituting methyl lanthionine for lanthionine to give two novel ring AB analogues.

To enable the SPPS of ring AB analogues it was first necessary to synthesise (Teoc/TMSE, Fmoc) lanthionine (**60**). This was initially carried out on a small scale using a published procedure. Adjustments to reaction conditions and purification procedures were made to facilitate scaling up of this route. The overall yield was significantly improved and time taken for synthesis was reduced, enabling the synthesis of lanthionine (**60**) on a medium scale. A short investigation was also carried out into whether cheaper starting materials could be used. This was successful, reducing the number of steps to (**60**) and avoiding two hazardous reactions.

With lanthionine (**60**) in hand the synthesis of simplified analogues of rings AB was then attempted. Three analogues were then designed in which Dha2 and Dhb5 were substituted with more stable residues: the biosynthetic precursors, threonine and serine to give (**129**); the saturated alkyl counterparts aminobutyric acid and alanine to give (**130**); α-aminoisobutyric acid (Aib), a known α-helix inducer and stabiliser to give (**131**).

Initially, a novel ring B analogue (**138**) was synthesised to check the SPPS methodology. A considerable amount of optimisation of this methodology was required for the synthesis of novel Thr/Ser analogue (**129**) which was successfully synthesised using double couplings, microwave conditions and a commercially available pseudoproline residue. Novel Abu/Ala analogue (**130)** was also synthesised successfully using the optimised methodology with both double coupling and microwave conditions. The use of Hmb amino acids was investigated in both analogues was not found to help synthesis. The synthesis of Aib/Aib analogue (**131**) was also attempted but was unsuccessful and time constraints meant this was not probed further. WT rings AB were obtained using a literature procedure for the preparation and digestion of commercially available nisin.

With two ring AB analogues and WT rings AB in hand, hybrid peptides were designed. The design centred around removing unstable dehydro residues and synthetically challenging lanthionine residues. The C-terminus of nisin was replaced with simpler, linear, pore forming peptides, mastoparan (**12**), pexiganan (**10**) and a peptoid (**15**) which could be synthesised by SPPS. Three different hinge region mimics were designed and incorporated into the SPPS of the pore forming, tail peptides and peptoid to give a set of seven novel tail + hinge region peptides and peptoids. In addition, mastoparan (**12**), pexiganan (**10**) and peptoid (**15**) were synthesised as controls.

Click chemistry methodology was then developed with a set of truncated model peptides. WT rings AB were alkylated with propargylamine to give (**110**) and click chemistry was used to couple this fragment with the tails + hinge region linkers to produce six novel hybrid peptides (**201**, **203-206**). Three of these (**203-205**) were taken on for initial biological testing using the agar well diffusion method and they were shown to have activity against a Gram positive indicator bacteria *Micrococcus luteus*. A further four hybrids (**201**, **203**, **205**, **206**) were taken on for further biological evaluation and a micro-dilution assay was developed to allow for MIC determination using minimal amount of peptide material. The hybrids were shown to have promising biological activity with MICs ranging from 0.75-12 μM, similar to the control tail peptides.

To build on this work, the synthesis of the hybrid peptides should be scaled up to give multi milligram quantities of the full set of target hybrids with both the longer Lys(N_3)-PEG4 linkers and the shorter N_3-PEG3 linkers. As the alkyl hinge region mimics, hybrids (**205**) and (**206**) were found to exhibit the poorest biological activity in the MIC assays and it would be reasonable not to include these in any further work. With greater quantities of hybrid peptides, it would then be desirable to screen for biological activity against a wider set of microbes including clinically relevant strains of MRSA and VRE. To test their drug-like properties, assays to test their stability and toxicity should be carried out.

Further optimisation of the hybrid peptide design would be pertinent to investigate (*Figure 6-1*). Possible modifications include shortening the hinge region further by using a diethylene glycol unit as the basis of a linker (**207**) or removing it altogether by clicking tail peptides directly to alkylated rings AB *via* 2-azidoaetic acid (**208**) Incorporation of the optimised hinge regions discussed in Chapter 1.3.8 would also be interesting to investigate by coupling the necessary residues to the N-terminus of the tail peptides (**209**). If the SPPS

of ring AB analogues is optimised further, full length analogues could be synthesised solely by SPPS and optimised hinge regions could be easily incorporated into the design (**210**). To further probe the role of ring C in the biological activity of nisin, a set of analogues that incorporate rings ABC could be synthesised. Rings ABC could be isolated using a chymotrypsin digest[183] of WT nisin (**211**) or synthesised using SPPS (**212**).

With analogues that are found to have good biological activity it may be useful to perform truncations to both the C-terminus and N-terminus of the tail peptides or peptoids to determine the minimum length necessary for activity.

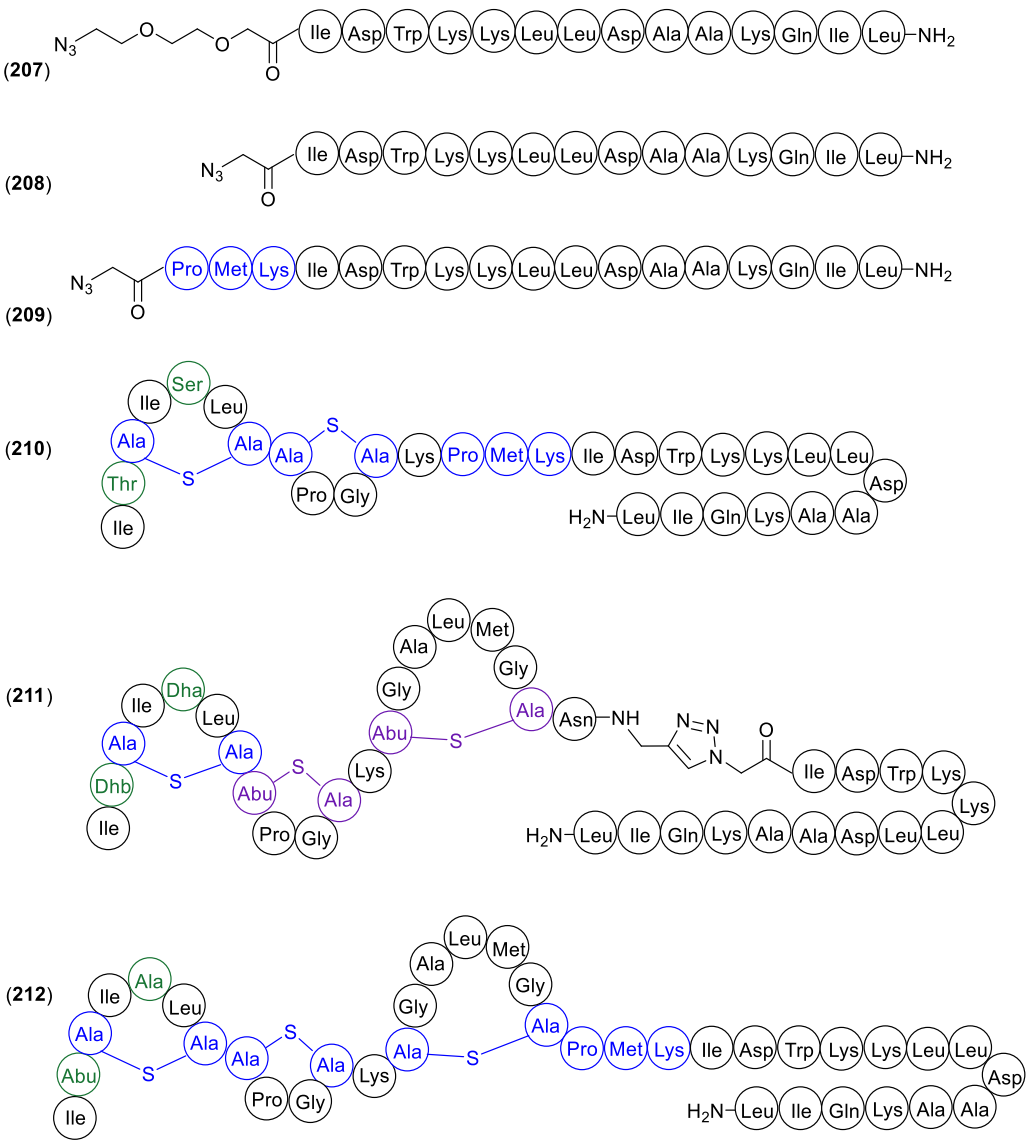


Figure 6-1: Modifications to hybrid peptide design

With the most promising hybrids further work would need to be carried out to elucidate their mechanism of action using LUVs encapsulating a fluorescent marker, both incorporating lipid II and without, to determine the extent of pore formation and lipid II binding.[186] These hybrids could also be evaluated for their selectivity, using haemolysis assays and their toxicity, using mammalian cell toxicity studies. Serum stability assays could help to predict if the simplified structures would be more stable *in vivo*.

Returning to the work carried out on ring AB analogues, the synthesis of the two novel analogues (**129**) and (**130**) should be scaled up to provide enough material for LUV, lipid II binding studies to assess the impact of the substitutions on binding to lipid II. A comparison of their NMR solution structures may also shed some light on this. If these results are promising the next step would be to incorporate the analogues into the best of the hybrid peptide design.

With the developed SPPS methodology and a further understanding of the hybrid peptides in place this work could be extended in a number of ways such as continuing the work of Zhou *et al*.[166] in attaching Gram negative active peptides to ring AB moieties or using ring AB moieties to improve selectivity of cell penetrating peptides that are known to have intracellular targets. This could be aided by designing a cleavable AB-peptide linkage.

7 Experimental

7.1 General

Reagents were purchased from chemical suppliers and were used as received without further purification. Custom, large scale synthesis of Fmoc-Cys-O^{t}Bu was carried out by Oxygen Healthcare, Cambridge, UK, which is part of Piramal Enterprises Ltd, Mumbai, India. Dry DMF, THF and CH_2Cl_2 were purchased as anhydrous solvents in Sureseal bottles from Sigma-Aldrich Co. Ltd. Brine refers to a saturated solution of sodium chloride in water and sodium bicarbonate refers to a saturated solution of sodium hydrogen carbonate in water. Ether refers to diethyl ether and petrol to petroleum ether fractions boiling between 40 and 60 °C. All water used was either distilled using an Elga Purelab Option R7 water purifier or used directly from a bottle of HPLC-grade water. All reactions were carried out in closed systems under Argon.

Thin layer chromatography (TLC) was performed on aluminium backed Sigma-Aldrich TLC plates with F_{254} fluorescent indicator. Developed plates were air dried and analysed under a UV light or by staining with the appropriate indicator. Normal phase flash column chromatography was carried out using silica gel (43-60 µm) supplied by Merck. Optical rotations were recorded using a Perkin Elmer 343 polarimeter and are reported in 10^{-1} deg cm^2 g^{-1}.

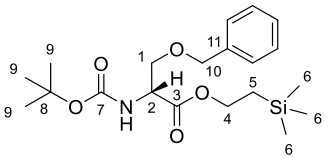
LRMS refers to low resolution mass spectrometry and HRMS refers to high resolution mass spectrometry. LRMS was performed on a Waters Acquity UPLC coupled to a SQD mass spectrometer using HPLC grade water and acetonitrile (both with 0.1% formic acid) as the solvents. HRMS electrospray ionization was determined using Waters LCT Premier XE instrumentation and Mass Lynx software.

NMR (^{1}H and ^{13}C) was performed on 300, 500 and 600 MHz AMX Bruker Spectrometers (as stated). The chemical shifts (δ) were given in units of ppm relative to tetramethylsilane (TMS), where δ(TMS) = 0 ppm. Data processing was carried out using ACD/NMR Academic Edition, Advanced Chemistry Development, Inc. For ^{1}H NMR, the multiplicity used for assignment is indicated by the following abbreviations: s = singlet, d = doublet, t = triplet, q = quadruplet, qn = quintet, m = multiplet, br = broad, app. d. = apparent doublet and the coupling constants (*J*) were measured in Hertz (Hz). ^{13}C NMR have been assigned using DEPT, HSQC and HMBC NMR as performed on 500 and 600 MHz AMX Bruker

Spectrometers. Deuterated chloroform ($CDCl_3$), dimethylsulfoxide (d_6-DMSO) and methanol (CD_3OD) were used as solvents (as stated) for all NMR analysis.

7.2 Experimental for Synthesis of Lanthionine

Boc-D-Ser(Bn)-OTMSE (113)[188]



Boc-Ser(Bn)-OH (**112**) (15.1 g, 48.8 mmol) and DMAP (1.19 g, 9.76 mmol) were dissolved in THF and the reaction mixture was cooled to -10 °C using a bath of sodium chloride and ice. TMSEOH (14.0 mL, 97.6 mmol) and DCC (13.1 g, 63.5 mmol) were added slowly. The reaction was warmed to room temperature and left stirring overnight. After this time the reaction mixture was concentrated *in vacuo* and the crude residue was dissolved in ethyl acetate (500 mL) and washed with $NaHCO_3$ (500 mL) and brine (200 mL). The organic layer was dried over $MgSO_4$, concentrated *in vacuo* and purified using flash column chromatography (10-30% ethyl acetate in petrol) to give (**113**) as an off white solid (16.6 g, 83%).

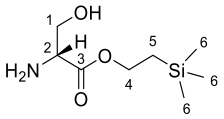
NMR: δ_H (600 MHz, CD_3OD) 0.05 (9H, s, Si(CH_3)$_3$), 0.96-1.0 (2H, m, $CH_2Si(CH_3)_3$), 1.46 (9H, s, C(CH_3)$_3$), 3.66-3.68 (1H, m, $ArCH_2OCH_2$), 3.85-3.87 (1H, m, 1 x $ArCH_2OCH_2$), 4.20-4.23 (2H, m, $CH_2CH_2Si(CH_3)_3$), 4.38-4.30 (1H, m, 1 x $ArCH_2OCH_2CH$), 4.47 (1H, d, *J* = 12.0 Hz, 1 x $ArCH_2OCH_2$), 4.54 (1H, d, *J* = 12.0 Hz, 1 x $ArCH_2OCH_2$), 5.39 (1H, d, *J* = 8.5 Hz, N*H*), 7.25-7.33 (5H, m, Ar).

δ_c (150 MHz, CD_3OD) -1.6 (C6), 17.3 (C5), 28.3 (C9), 54.1 (C2), 63.9 (C4), 70.0 (C1), 73.2 (C10), 79.9 (C8), 127.6, (CAr) 128.5 (CAr), 129.4 (CAr), 137.6 (C11), 155.5 (C3), 170.8 (C7).
NMR data is in agreement with the literature.[188]

LRMS: (ES$^+$) $[C_{20}H_{34}NO_5Si]^+$ calculated = 396.2; found *m/z* = 418.2 $[M+Na]^+$

$[\alpha]^{20}_D$ = - 8.2 (c = 10 mg mL^{-1}, $CHCl_3$)
[lit[188] $[\alpha]^{20}_D$ = - 7.6 (c = 10 mg mL^{-1}, $CHCl_3$)]

H-D-Ser-OTMSE (114)[188]



Pd/C (10%, 0.50 g) was added to a solution of Boc-D-Ser(Bn)-OTMSE (**113**) (4.00 g, 9.77 mmol) in ethanol (100 mL) and the flask was placed under a hydrogen atmosphere for 24 h. After this time, the reaction was filtered over a bed of celite and the solvent was removed *in vacuo*.

The crude product was treated with a solution of TFA (5 mL) and CH_2Cl_2 (5 mL) and left to stir for 2 h. After this time, the reaction was concentrated *in vacuo* and excess toluene was added and removed *in vacuo*. This was repeated twice and was followed by CH_2Cl_2. Flash column chromatography was used to purify the residue (2-5% MeOH in CH_2Cl_2) to give (**114**) as a colourless oil (1.73 g, 86%).

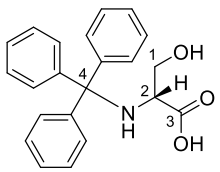
NMR: δ_H (600 MHz, CD_3OD) 0.06 (9H, s, Si(CH_2)$_3$), 0.97-1.10 (2H, m, $CH_2Si(CH_3)_3$), 3.91-4.06 (3H, m, $CHCH_2OH$), 4.31-4.36 (2H, m, $CO_2CH_2CH_2Si(CH_3)_3$).

δ_c (150 MHz, CD_3OD) -1.5 (C6), 18.4 (C5), 56.3 (C2), 60.8 (C4), 66.2 (C1), 169.2 (C3).
NMR data is in agreement with the literature.[188]

LRMS: (ES$^+$) $[C_8H_{20}NO_3Si]^+$ calculated = 206.1; found *m/z* = 228.0 $[M+Na]^+$

$[\alpha]^{20}_D$ = - 34.2 (c = 7.5 mg mL^{-1}, MeOH)
[lit[188] $[\alpha]^{20}_D$ = - 41.0 (c = 7.3 mg mL^{-1}, MeOH)]

H-Trt-D-Ser-OH (128)[234]



Trimethyl silyl chloride (18.7 mL, 147 mmol) was added to D-serine (**127**) (5.00 g, 47.6 mmol) in anhydrous CH_2Cl_2. The reaction mixture was heated at reflux for 40 min and then left to cool to room temperature. Triethylamine (20.6 mL, 147 mmol) was diluted in CH_2Cl_2 and added dropwise to the reaction mixture. The reaction was again heated at reflux for a further hour after which it was cooled to 0 °C. Anhydrous methanol (1.93 mL, 47.6 mmol) was added dropwise and the reaction mixture was warmed to room temperature. A further

portion of triethylamine was added (6.63 mL, 47.6 mmol) and then triphenylmethyl chloride (13.2 g, 47.6 mmol) was added portion-wise and the reaction was left to stir overnight. Methanol and triethylamine were added until all solids had dissolved and the reaction mixture was concentrated *in vacuo*. The crude residue was dissolved in ethyl acetate and washed with 5% citric acid (2 x 500 mL). The organic layers were dried over $MgSO_4$ and concentrated *in vacuo*. Flash column chromatography was used to purify the crude material (0-15% MeOH in CH_2Cl_2) to give (**128**) as an off-white powder (6.30 g, 38%).

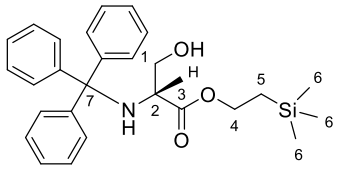
δ_H (600 MHz, CD_3OD) 3.02-3.05 (1 H, m, 1 x CHC*H*$_2$OH), 3.26-3.32 (2H, m, C*H*CH_2OH & 1 x CHC*H*$_2$OH),3.55 (1H, br s, N*H*), 7.21(3H, t, *J* = 7.0 Hz, Ar), 7.26 (6H, m, *J* = 7.0 Hz, Ar), 7.50 (6H, d, *J* = 7.0 Hz, Ar).

δ_c (150 MHz, CD_3OD) 58.6 (C2), 62.6 (C1), 71.9 (C4), 127.4 (CAr), 128.5 (CAr), 128.6 (CAr), 144.7 (CAr), 174.3 (C3).
NMR data is in agreement with literature.[234]

LRMS: (ES^-) $[C_{22}H_{20}NO_3]^-$ calculated: 346.1; found *m/z* = 346.3 $[M-H]^-$, 693.3 $[2M-H]^-$

$[\alpha]^{20}_D$ = -12.9 (3.5 mg mL^{-1}, EtOH)

Trt-D-Ser-OTMSE (115)[188]



Method 1 - from H-Ser-OTMSE (**114**)
Triethylamine (2.88 mL, 20.5 mmol) was added to a solution of H-Ser-OTMSE (**114**) (2.10 g, 10.3 mmol) in DMF (200 mL) and the reaction mixture was cooled to 0 °C. To this, trityl chloride (2.58 g, 9.24 mmol) was added and the flask was warmed to room temperature. After 24 h, the mixture was washed with water (2 x 200 mL) and extracted with CH_2Cl_2 (2 x 200 mL) and flash column chromatography was used to purify the residue (0-1% MeOH in CH_2Cl_2) to give (**115**) as a colourless oil (2.24 g, 49%).

Method 2 (from Trt-D-Ser-OH) (**128**)

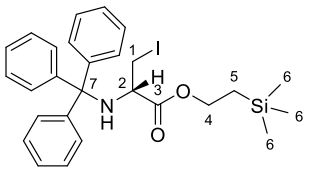
Trt-D-Ser-OH (**128**) (5.00 g, 14.4 mmol) and 2-(trimethylsilyl)ethanol (22.7 mL, 158 mmol) were dissolved in CH_2Cl_2 (5 mL) and the reaction mixture was cooled to 0 °C. DMAP (7.04 g, 57.6 mmol) and EDCI (3.31 g, 17.3 mmol) were added to the reaction mixture which was then allowed to warm to room temperature and stirred for 24 h. After this time, excess CH_2Cl_2 (100 mL) was added and the reaction mixture was washed aqueous citric acid solution (5% w/v, 150 mL) and brine (150 mL). The organic layer was dried over $MgSO_4$ and the solvent was removed *in vacuo*. The crude residue was purified by flash column chromatography (0-1% MeOH in CH_2Cl_2) to give (**115**) as a colourless oil (4.45g, 69%).

NMR: δ_H (600 MHz, $CDCl_3$) 0.01 (9H, s, Si(CH_3)$_3$), 0.76-0.81 (2H, m, CH_2Si(CH_3)$_3$), 3.49-3.54 (2H, m, CHCH_2OH), 3.67-3.70 (2H, m, $CO_2CH_2CH_2Si(CH_3)_3$), 3.76-3.80 (1H, m, TrtNHCHCH$_2$), 7.18-7.21 (3H, m, Ar), 7.25-7.31 (6H, m, Ar), 7.48-7.50 (6H, m, Ar).

δ_c (150 MHz, $CDCl_3$) -1.6 (C6), 17.0 (C5), 57.8 (C2), 63.4 (C4), 64.8 (C1), 70.0 (C7), 126.6 (CAr), 127.2 (CAr), 127.9 (CAr), 128.7 (CAr), 145.6 (CAr), 173.6 (C3).
NMR data is in agreement with the literature.[188]

LRMS: (ES$^+$) $[C_{28}H_{36}NO_3Si]^+$ calculated = 462.7; found *m/z* = 485.7 $[M+Na]^+$

2-(Trimethylsilyl)ethyl (*S*)-3-iodo-2-(tritylamino)propanoate (116)[188]



The protected serine (**115**) (1.20 g, 2.81 mmol) and PPh_3 (1.10 g, 4.21 mmol) were dissolved in anhydrous CH_2Cl_2 (3 mL) at room temperature and left stirring for ten minutes. The reaction mixture was then cooled to -10 °C and DEAD (77.0 µL, 4.21 mmol) was added dropwise. After another five minutes MeI (26.0 µL, 4.21 mmol) was added and the reaction mixture was left stirring at -10 °C for 3 h. The reaction mixture was purified directly by flash column chromatography (10-25% ethyl acetate in hexane) to give the iodinated compound (**116**) as a clear oil (1.05 g, 77%).

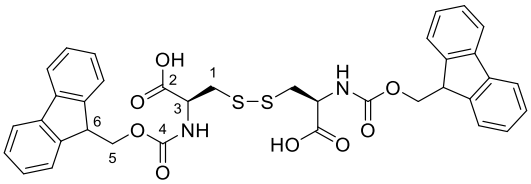
NMR: δ_H (600 MHz, $CDCl_3$) 0.01 (9H, s, $SiCH_3)_3$, 0.77-0.87 (2H, m, $CH_2Si(CH_3)_3$), 2.91 (1H, d, *J* = 9.0 Hz, N*H*), 3.21 (1H, dd, *J* = 7.0, 9.5 Hz, TrtNHCHCH_2I), 3.33 (1H, dd, *J* = 3.0, 9.5 Hz, TrtNHCHCH_2I), 3.34-3.46 (1H, m, TrtNHC*H*CH_2I), 3.7 (1H, td, *J* = 6.0, 11.5 Hz, $CO_2CH_2CH_2Si$), 3.82 (1H, td, *J* = 6.0, 11.5 Hz, $CO_2CH_2CH_2Si$), 7.19-7.24 (3H, m, Ar), 7.28-7.30 (6H, m, Ar), 7.51-7.52 (6H, m, Ar).

δ_C (150 MHz, $CDCl_3$) -1.6 (C6), 10.2 (C1), 17.2 (C5), 56.1 (C2), 63.6 (C4), 71.1 (C7), 126.6 (CAr), 128.0 (CAr), 128.7 (CAr), 145.7 (CAr), 172.4 (C3).
NMR data is in agreement with the literature.[188]

LRMS: (ES^+) $[C_{27}H_{33}INO_2Si]^+$ calculated = 558.1; found *m/z* = 580.0 $[M+Na]^+$

$[\alpha]^{20}_D$ = -5.8 (c = 6.6 mg mL^{-1}, $CHCl_3$)
[lit[188] $[\alpha]^{20}_D$ = -8.1 (c = 5.2 mg mL^{-1}, $CHCl_3$)]

(Fmoc-Cys-OH)$_2$ (120)[235]



Method 1 from (Fmoc-Cys-O^{t}Bu)$_2$ (**118**)
(Fmoc-Cys-OtBu)$_2$ (10.0 g, 12.6 mmol) was dissolved in 10 mL TFA and 10 mL CH_2Cl_2. After 3 h the solvent was removed *in vacuo* to give (**120**) as an off white solid (11.5 g, 99%).

Method 2 from L-Cystine (**117**)
Sodium hydroxide (3.33 g, 83.2 mmol) was dissolved in 100 mL of water. THF (100 mL) was added and the mixture was cooled to 0 °C before the addition of cystine (5.00 g, 20.8 mmol). The reaction mixture was left overnight and after this time the THF was removed *in vacuo* and the reaction mixture was acidified with 1.0 M HCl. The white precipitate could then be collected by filtration and the solid was re-suspended in ethyl acetate and left in the fridge overnight. The precipitate was again filtered and washed with portions of cold ethyl acetate to give an off white solid (**120**) (10.3 g, 52%).

NMR: δ_H (600 MHz, CD_3OD) 3.02 (2H, dd, *J* = 14.0, 9.5 Hz, CHCH_2S), 3.28 (2H, m, CHCH_2S), 4.19, (2H, t, *J* = 7.0 Hz, 2 x C*H*CH_2Fmoc), 4.26-4.33 (4H, m, 2 x CHCH_2Fmoc), 4.54 (2H, dd, *J* =

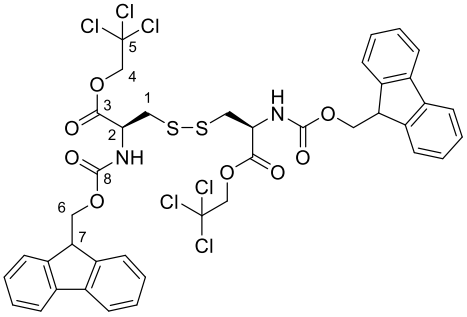
9.5, 4.5 Hz, C*H*CH_2S), 7.27 (4H, t, *J* = 7.5 Hz, Ar), 7.33-7.37 (4H, m, Ar), 7.65 (4H, d, *J* = 7.5 Hz, Ar), 7.75-7.76 (4H, m, Ar).

δ_c (150 MHz, CD_3OD) 41.1 (C1), 48.3 (C6), 54.7 (C3), 68.2 (C5), 126.3 (CAr), 121.0 (CAr), 126.6 CAr) 128.9 (CAr), 142.6 (CAr), 145.4 (CAr), 158.5 (C4), 174.0 (C3).
NMR data is in agreement with the literature.[235]

LRMS: (ES^-) $[C_{36}H_{31}N_2O_8S_2]^-$ calculated = 683.2; found *m/z* = 683.2 $[M-H]^-$

$[\alpha]^{25}_D$ = -65.4 (c = 9.0 mg mL^{-1}, MeOH)
[lit[189] $[\alpha]^{25}_D$ = -79.6 (c = 8.9 mg mL^{-1}, MeOH)]

(Fmoc-Cys-OTce)$_2$ (121)[195]



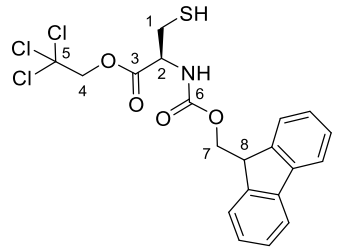
(Fmoc-Cys-OH)$_2$ (**120**) (5.00 g 7.30 mmol) and *p*-toluenesulfonic acid (3.33 g, 190 mmol) were dissolved in benzene (250 mL). 2,2,2-Trichloroethanol (2.62 g, 17.5 mmol) was added. The reaction was stirred at 110 °C under reflux for 24 h with a Dean-Stark trap attached. After this time the reaction was cooled to 0 °C and any unreacted acid was removed by filtration. The solvent was removed *in vacuo* and the crude residue was dissolved in chloroform (200 mL), washed with water (200 mL) and brine (200 mL). Flash column chromatography was used to give the title compound (**121**) as a white solid (6.02 g, 87%).

NMR: δ_H (600 MHz, $CDCl_3$) 3.14-3.31 (4H, m, CHCH_2S), 4.19-4.23 (2H, m, 2 x C*H*CH_2Fmoc), 4.41-4.57 (4H, m, 2 x CHCH_2Fmoc), 4.72-4.85 (6H, m, CH_2Cl_3 & C*H*CH_2S), 5.74-5.80 (2H, m, N*H*), 7.30-7.32 (4H, m, Ar), 7.39-7.49 (4H, m, Ar), 7.56-7.64 (4H, m Ar), 7.75-7.80 (4H, m, Ar).

δ_C (150 MHz, $CDCl_3$) 40.7 (C1), 47.0 (C7), 53.3 (C2), 67.4 (C6), 74.8 (C4), 94.1 (C5), 120.0 (CAr), 125.0 (CAr), 127.1 (CAr), 127.8 (CAr), 141.3 (CAr), 143.5 (CAr), 155.7 (C8), 168.9 (C3).
NMR data is in agreement with the literature.[195]

LRMS: (ES$^+$) $[C_{40}H_{35}Cl_6N_2O_8S_2]^+$ calculated = 945.0; found *m/z* = 948.68 $[M+H]^+$

Fmoc-Cys-OTce (122)[195]



Method 1

Triphenylphosphine (521 mg, 2.18 mmol), 2-mercaptoethanol (185 mg, 2.37 mmol) and water (89.0 µg, 4.95 mmol) were added to (Fmoc-Cys-OTce)$_2$ (451 mg, 495 µmol) in THF (100 mL). The reaction was stirred at 50 °C for 5 h. After this time the reaction mixture was concentrated *in vacuo* and flash column chromatography (9% ethyl acetate in hexane) was used to purify the crude residue directly giving the thiol (**122**) as a white solid (298 mg, 66%).

Method 2

Protected cystine (**107**) (3.79 g, 4.00 mmol) was dissolved in CH_2Cl_2 (250 mL). DTT (740 mg, 4.79 mmol) and then triethylamine (670 µL, 4.79 mmol) were added and the reaction was left to stir for 4 h. After this time excess CH_2Cl_2 (250 mL) was added and the reaction mixture was washed with sodium bicarbonate (2 x 200 mL), water (2 x 200 mL) and brine (1 x 200 mL). The organic layer was dried over $MgSO_4$ and concentrated *in vacuo* to give (**122**) as a white solid (3.49 g, 92%).

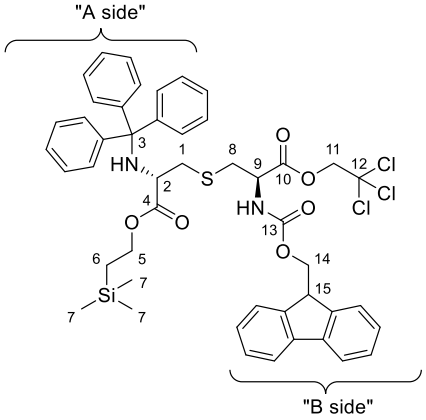
NMR: δ_H (600 MHz, $CDCl_3$) 1.47 (1H, t, *J* = 9.0 Hz, SH), 3.02-3.07 (1H, m, CHC*H*$_2$S), 3.14-3.18 (1H, m, CHC*H*$_2$S), 4.25 (1H, t, *J* = 7.0 Hz, C*H*CH$_2$Fmoc), 4.46 (2H, d, *J* = 7.0 Hz, CHC*H*$_2$Fmoc), 4.74 (1H, d, *J* = 12.0 Hz, C*H*$_2$CCl$_3$), 4.83-4.85 9 (1H, m, C*H*CH$_2$S), 4.94 (1H, d, *J* = 12.0 Hz, C*H*$_2$CCl$_3$), 5.71 (1H, d, *J* = 7.5 Hz, NH), 7.33-7.35 (2H, m, Ar), 7.43 (2H, t, *J* = 7.5 Hz, Ar), 7.62 (2H, d, *J* = 7.0 Hz, Ar), 7.79 (2H, d, *J* = 8.0 Hz, Ar).

δ_C (150 MHz, $CDCl_3$) 27.0 (C1), 47.1 (C8), 55.1 (C2), 67.3 (C7), 74.6 (C4), 94.2 (C5), 120.1 (CAr), 125.0 (CAr), 127.1 (CAr), 127.8 (CAr), 141.3 (CAr), 143.5 (CAr) 143.7 (CAr), 155.6 (C6), 168.7 (C3).

NMR data is in agreement with the literature.[195]

LRMS: (ES$^+$) $[C_{20}H_{19}Cl_3NO_{4S}]^+$ calculated = 474.0; found *m/z* = 475.9 $[M+H]^+$

2,2,2-Trichloroethyl *N*-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-*S*-((*S*)-3-oxo-3-(2-(trimethylsilyl)ethoxy)-2-(tritylamino)propyl)-*L*-cysteinate (123)[188]



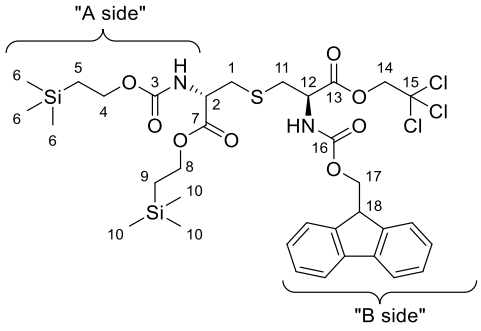
The protected cysteine (**122**) (831 mg, 1.82 mmol) and 2-(trimethylsilyl)ethyl (*S*)-3-iodo-2-(tritylamino)propanoate (**116**) (1.01 g, 1.82 mmol) were dissolved in DMF (100 mL) and the reaction mixture was cooled to 0 °C. Caesium chloride (592 mg, 1.82 mmol) was added and the reaction mixture was left stirring at 0 °C for 3 h. After this time, excess ethyl acetate was added and the mixture was washed with citric acid (100 mL) and water (2 x 500 mL). The combined organic layers were concentrated *in vacuo* and the residue was purified using flash column chromatography (5% ethyl acetate in hexane) to give the coupled product (**123**) as a clear oil (922 mg, 56%).

NMR: δ_H (600 MHz, $CDCl_3$) 0.01 (9H, s, Si(C*H*$_3$)$_3$), 0.72-0.83 (2H, m, C*H*$_2$Si(CH$_3$)$_3$), 2.75 (1H, dd, *J* = 7.5, 13.5 Hz, H$_\beta$ A side), 2.92 (1H, dd, *J* = 4.5, 13.5 Hz, *H*$_\beta$ A side), 3.02 (1H, dd, *J* = 6.5, 14.0 Hz, *H*$_\beta$ B side), 3.15 (1H, dd, *J* = 4.5, 14.0, *H*$_\beta$ B side), 3.51-3.53 (1H, m, *H*$_\alpha$ A side), 3.61 (1H, td, *J* = 5.5, 11.5 Hz, 1 x $CO_2CH_2CH_2Si$), 3.76 (1H, td, *J* = 5.5, 11.5, 1 x $CO_2CH_2CH_2Si$), 4.25 (1H, t, *J* = 7.0 Hz, C*H*Fmoc), 4.38-4.44 (2H, m, C*H*$_2$Fmoc), 4.72 (1H, d, *J* = 12.0 Hz, 1 x C*H*$_2$CCl$_3$), 4.75-4.78 (1H, m, *H*$_\alpha$ B side), 4.85 (1H, d, *J* = 12.0 Hz, 1 x C*H*$_2$CCl$_3$), 5.72 (1H, d, *J* = 8.5 Hz, N*H*Fmoc), 7.19 (3H, t, *J* = 7.0 Hz, Ar), 7.26-7.28 (5H, m, Ar), 7.30-7.34 (3H, m, Ar), 7.4-7.43 (2H, m, Ar), 7.5 (6H, d, *J* = 7.5 Hz, Ar), 7.62-7.65 (2H, m, Ar), 7.78 (2H, d, *J* = 7.5 Hz, Ar).

δ_C (150 MHz, $CDCl_3$) -1.6 (C7), 17.0 (C6), 34.6 (C8), 37.6 (C1), 47.0 (C15), 53.6 (C9), 56.3 (C2), 63.4 (C5), 67.4 (C14), 71.2 (C3), 74.6 (C11), 94.3 (C12), 120.0 (CAr), 125.2 (CAr), 126.6 (CAr), 127.1 (CAr), 127.7 (CAr), 127.9 (CAr), 128.7 (CAr), 129.7 (CAr), 141.3 (CAr), 143.7 CAr), 145.7 (CAr), 155.8 (C13), 169.3 (C10), 173.6 (C4).
NMR data is in agreement with the literature.[188]

LRMS: (ES^+) $[C_{47}H_{50}Cl_3N_2O_6SSi]^+$ calculated = 903.2; found *m/z* = 905.0 $[M+H]^+$

2,2,2-Trichloroethyl (8*S*,12*R*)-12-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-2,2-dimethyl-6-oxo-8-((2-(trimethylsilyl)ethoxy)carbonyl)-5-oxa-10-thia-7-aza-2-silatridecan-13-oate (124)[188]



The coupled lanthionine (**123**) (1.16 g, 1.19 mmol) was dissolved in CH_2Cl_2 (40 mL) and TFA (4.1 mL) and TIPS (1 mL) were added. The reaction was left stirring at room for 3 h. After this time, excess chloroform was added and the organic layer was washed with sodium bicarbonate (2 x 200 mL) and brine (200 mL). The combined organic layers were dried over $MgSO_4$ and concentrated *in vacuo*. The crude residue was dissolved in 1,4-dioxane (180 mL) which was followed by addition of DMAP (145 mg, 1.19 mmol) and 1-[2-(trimethylsilyl)ethoxycarbonyloxy]pyrrolidine-2,5-dione (1.53 g, 5.90 mmol). The reaction was left to stir at room temperature overnight. Excess chloroform was added and the organic layer was washed with brine (200 mL) and then dried over $MgSO_4$, concentrated *in vacuo* and purified by flash column chromatography (10-25% ethyl acetate in hexane) to give the title product (**124**) as a clear oil (566 mg, 58%).

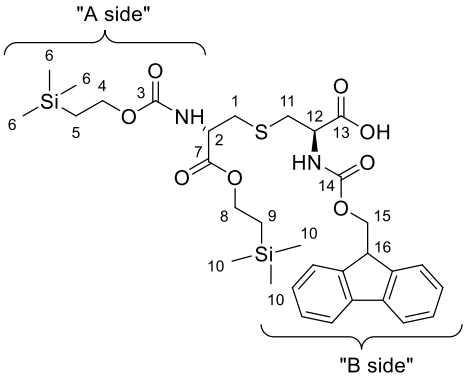
NMR: δ_H (600 MHz, $CDCl_3$) 0.02 (18H, app. d, $Si(CH_3)_3$), 0.98-1.01 (4H, m, $CH_2Si(CH_3)_3$), 2.95-2.96 (1H, m, H_β B side), 3.06-3.15 (3H, m, 1 x H_β B side, 2 x H_β A side), 4.16-4.26 (5H, m, CHFmoc, CH_2CH_2Si), 4.38-4.44 (2H, m, CH_2Fmoc), 4.57 (1H, s, H_α A side), 4.77-4.79 (2H, m, CH_2CCl_3, H_α B side), 4.86-4.88 (1H, m, CH_2CCl_3), 5.48 (1H, s, NHFmoc), 5.93 (1H, s, NHTeoc),

7.33 (2H, t, *J* = 7.5 Hz, Ar), 7.41 (2H, t, *J* = 7.5, Ar), 7.63-7.65 (2H, m, Ar), 7.77 (2H, d, *J* = 7.5, Ar).

δ_C (150 MHz, $CDCl_3$) -1.6,-1.5 (C6, C10), 17.4, 17.6 (C5, C9), 35.3, 36.1 (C1,C11) 47.0, (C18) 50.9 (C2), 53.8 (C12), 63.8, 64.6 (C4, C8), 67.7 (C17), 74.6 (C14), 94.2 (C15), 120.0 (CAr), 125.1 (CAr), 125.2 (CAr), 127.1 (CAr), 127.8 (CAr), 141.3 (CAr), 143.6 (CAr), 143.7 (CAr), 156.2 (C16), 168.8 (C3), 169.0 (C7) 170.6 (C13).
NMR data is in agreement with the literature.[188]

LRMS: (ES^+) $[C_{34}H_{48}Cl_3N_2O_8SSi_2]^+$ calculated = 805.2; found *m/z* = 829.3 $[M+Na]^+$

(8*S*,12*R*)-12-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-2,2-dimethyl-6-oxo-8-((2-(trimethylsilyl)ethoxy)carbonyl)-5-oxa-10-thia-7-aza-2-silatridecan-13-oic acid (60)[188]



Zinc dust (450 mg, 6.89 mmol) was added slowly to a solution of the protected lanthionine (**124**) (565 mg, 689 µmol) in THF (100 mL). NH_4OAc solution (1.0 M, 5 mL) was then added and the reaction was left to stir under nitrogen overnight. After this time the reaction was filtered and concentrated *in vacuo*. The residue was then dissolved in excess chloroform and washed with brine (200 mL). The organic layer was dried over $MgSO_4$ and concentrated *in vacuo* to give a crude residue that was purified by flash column chromatography (2, 5% MeOH in CH_2Cl_2) to give the protected lanthionine (**60**) (298 mg, 66%).

NMR: δ_H (600 MHz, $CDCl_3$) 0.00 (18H, app. d, $Si(CH_3)_3$), 0.88-0.93 (4H, m, $CH_2Si(CH_3)_3$), 2.93-2.98 (3H, m, 2 x H_β B side, 1x H_β A side), 3.11-3.16 (1H, m, H_β A side), 4.04-4.14 (4H, m, 2x CH_2CH_2Si,), 4.19-4.21 (2H, m, CH_2Fmoc), 4.32-4.41 (3H, m, H_α A side, H_α B side, CHFmoc), 7.25 (2H, t, *J* = 7.5 Hz, Ar), 7.33 (2H, t, *J* = 7.5, Ar), 7.64 (2H, d, *J* = 7.0 Hz, Ar), 7.73 (2H, d, *J* = 7.0 Hz, Ar).

δ_C (150 MHz, CDCl3) -1.3,-1.24 (C6, 10), 18.3, 18.7 (C5, 9), 35.5 (C11), 36.7 (C1), 48.5 (C16), 55.8, 57.2 (C2, 12), 64.5, 65.0 (C4, 8), 68.4 (C15), 94.2 (CAr), 121.1 (CAr), 126.5 (CAr), 126.6 (CAr), 128.3 (CAr), 128.4 (CAr), 128.9 (CAr), 142.6 (CAr), 145.4 (CAr), 158.6 (C14), 158.8 (C3), 172.8 (C7), 178.6 (C13).
NMR data is in agreement with the literature.[188]

LRMS: $(ES)^-$ $[C_{32}H_{45}N_2O_8SSi_2]^-$ calculated = 673.2; found *m/z* = 673.4 $[M-H]^-$.

$[\alpha]^{25}_D$ = +3.6 (c = 10 mg mL^{-1}, MeOH)

7.3 Experimental for Peptide Synthesis

7.3.1 General Methods

Peptides were synthesised using an automated peptide synthesiser or by hand, manually. In both cases synthesis was carried out in 5 mL syringes with frits, purchased from Multisyntech GmbH. Microwave couplings and click chemistry were carried out using a Personal Chemistry Smith Creator Microwave Assisted Organic Synthesizer loaded with either 0.5, 2 or 5 mL reaction vials as purchased from Biotage. HPLC grade DMF purchased from Sigma Aldrich was used as the primary solvent throughout peptide synthesis. Peptides were centrifuged using an Eppendorf Centrifuge 5810 R and were lyophilised using an VirTis Benchtop Pro freeze dryer.

Hmb-protected amino acids and Fmoc-Ile-Ser[ψ(^{Me,Me})pro]-OH were purchased from Merck.

Method 1: Automated Peptide Synthesis

Peptides were synthesised on a MultiSynTech Syro Peptide Synthesiser (model MP-60). The peptide synthesiser contains an agitation block which held 5 mL syringes with frits connected to a vacuum pump to remove solvents. Pre-loaded Rink amide resins (100-200 mesh) purchased from Merck KGaA were used in all cases of automated peptide synthesis. The total volume of all reagents in each step was 1.5 mL and all reagents were dissolved in HPLC grade DMF.

*Fmoc Deprotectio*n: A solution of piperidine in DMF (40% v/v, 1.5 mL) was added to the syringe containing an N-terminal Fmoc-protected peptide. The mixture was agitated for 20

sec every minute for a total of 3 min. The reagents were removed by vacuum filtration and the resin was washed with DMF (6 x 1.5 mL). A second portion of piperidine in DMF (40% v/v, 0.75 mL) was added to the syringe followed by DMF (0.75 mL) to give a final solution of 20% v/v of piperidine in DMF. The mixture was agitated for 20 sec every minute for a total of 10 min. The reagents were removed by vacuum filtration and the resin washed with DMF (6 x 1.5 mL)

Amino Acid Coupling: Fmoc protected amino acid (4 eq.), HBTU (4 eq.) and DIPEA (4 eq.) were added to the reaction syringe. The mixture was agitated for 20 sec every minute for a total of 40 min. The reagents were removed by vacuum filtration and the resin was washed with DMF (4 x 1.5 mL).

Method 2: Manual Peptide Synthesis

Manual peptide synthesis was carried out using an IKA KS130 basic platform shaker to agitate the solutions. Reactions were carried out in 5 mL syringes with frits which were evacuated by hand after every reaction or wash. Amino acid reagent weights and concentrations were calculated based on quantity and loading of the resin and were added to the syringe by hand. All standard amino acids were pre-activated with the coupling reagents and base before being added to the resin. The total volume of all reagents in each step was 1.5 mL and all reagents were dissolved in HPLC grade DMF unless otherwise stated.

Fmoc Deprotection: A solution of piperidine in DMF (40% v/v, 1.5 mL) was added to the syringe containing an N-terminal Fmoc-protected peptide. The mixture was agitated for a total of 3 min. The reagents were removed by vacuum filtration and the resin was washed with DMF (6 x 1.5 mL). A second portion of piperidine in DMF (40% v/v, 0.75 mL) was added to the syringe followed by DMF (0.75 mL) to give a final solution of 20% v/v of piperidine in DMF. The mixture was agitated for a total of 10 min. The reagents were removed by vacuum filtration and the resin washed with DMF (6 x 1.5 mL)

Standard Amino Acid Coupling: Fmoc-protected amino acids (4 eq.), HOAt (5 eq.), PyAOP (5 eq.) and DIPEA (10 eq.) were dissolved in DMF (1.5 mL) and added to the reaction syringe. The mixture was agitated for a total of 1 h and the reagents were then removed by filtration and the resin washed with DMF (5 x 1.5 mL). If double coupling was carried out the process was then repeated.

Teoc/TMSE Deprotection: 1 mL of TBAF (1.0 M, THF) and 1 mL of DMF were added to the reaction syringe. The mixture was agitated for 1 h. After this time the reagents were removed by filtration and the resin washed with DMF (5 x 1.5 mL).

Lanthionine Coupling: Lanthionine (**60**) (3 eq.), HOAt (5 eq.), PyAOP (5 eq.) and DIPEA (10 eq.) were dissolved in DMF (4 mL) and added the reaction syringe. The resin and reagents were then transferred to a microwave vial which was irradiated for 5 min at 60 °C and then left to stir at room temperature for a further 1 h. The resin and reagents were transferred back to the reaction syringe and the resin was washed with DMF (5 x 1.5 mL).

On-resin Cyclisation: HOAt (5 eq.), PyAOP (5 eq.) and DIPEA (10 eq.) were dissolved in DMF (4 mL) and added to the reaction syringe. The resin and reagents were then transferred to a microwave vial which was irradiated for 5 min at 60 °C and then left to stir at room temperature for a further 1 h. The resin and reagents were transferred back to the reaction syringe and the resin was washed with DMF 5 x (1.5 mL).

Hmb-Protected Amino Acid Coupling: Hmb-protected amino acid (5 eq.). HBTU (5 eq.) and DIPEA (10 eq.) were dissolved in DMF (1.5 mL) and added to the reaction syringe. The mixture was agitated for a total of 1 h and the reagents were then removed by filtration and the resin washed with DMF (5 x 1.5 mL). If double coupling was carried out the process was then repeated.

Symmetrical Anhydride coupling: The resin was first washed with CH_2Cl_2 (5 x 1.5 mL). The required symmetric anhydride (10 eq.) was dissolved in CH_2Cl_2 (1.5 mL) and added to the reaction syringe. The mixture was agitated for a total of 1 h and the reagents were then removed by filtration and the resin washed with DMF (5 x 1.5 mL). If double coupling was carried out the process was then repeated.

Method 3: Automated Peptoid Synthesis

Peptoids were synthesised in a similar manner to peptides using an automated peptide synthesiser as before. In addition to DMF, reagent grade NMP purchased from Sigma Aldrich was also used. The synthesiser was reprogrammed to add the non-amino acid reagents and both NMP and DMF were used as solvents. Bromoacetic acid was used as a 1.2 M solution in DMF and boc-butanediamine and MBA were both used as 1 M solutions in NMP. DIC was used neat.

Fmoc Deprotection of Resin: The Fmoc deprotection protocol for automated peptide synthesis was used.

Bromoacetic Acid Coupling: Bromoacetic acid (20 eq.) and DIC (25 eq.) were added to the reaction syringe and the mixture was agitated for 20 sec every minute for a total of 1 h. The reagents were removed by vacuum filtration and the resin was washed with DMF (3 x 1.5 mL) and NMP (3 x 1.5 mL).

Amine Coupling: The required amine (25 eq.) was added to the reaction syringe and the mixture was agitated for 20 sec every minute for a total of 1 h. The reagents were removed by vacuum filtration and the resin was washed with NMP (3 x 1.5 mL) and DMF (3 x 1.5 mL).

Cleavage and Purification

Cleavage and purification procedures were the same for both peptides and peptoids made manually and those made using the peptide synthesiser.

Cleavage from the Resin: Peptides and peptoids were washed with DMF (5 x 1.5 mL), CH_2Cl_2 (5 x 1.5 mL), methanol (5 x 1.5 mL) and diethyl ether (5 x 1.5 mL) and dried in desiccator for 30 min. A solution of 96.5% TFA, 2.5% water and 1% TIPS (1 mL) was added to the reaction syringe and the mixture was agitated for 40 min on the platform shaker. The cleavage solution was removed by filtration and the filtrate was transferred to a 15 mL Falcon tube containing 13 mL of diethyl ether to give a milky white precipitate. Another aliquot (1 mL) of the cleavage solution was added to the reaction syringe and the mixture was agitated for a further 20 min on the platform shaker. The cleavage solution was again removed by filtration and transferred to the same Falcon tube. The Falcon tube was centrifuged for 10 min at 4000 rpm at 0 °C after which the diethyl ether was carefully decanted. This process was repeated with a further 2 aliquots of fresh diethyl ether (13 mL) after which the peptide was re-dissolved in water and lyophilised.

HPLC Purification: Peptides and peptoids were analysed and purified via reverse phase HPLC using a Dionex system with a PDA-100 photodiode array detector, a model ASI-100 automated sample injector and Chromeleon Software version 2.0. Preparative purification was performed using an ACE 5 C8-300 column (150 x 10 mm), a Dr Maisch Reprosil Gold 200 C8 (150 x 10 mm) or a Phenomenex Onyx monolithic C18 column (100 x 3 mm) with UV detection at 214 and 254 nm. Conditions varied between peptides and are reported as

such. The mobile phase is quoted as a percentage of solvent B (acetonitrile with 0.1% TFA) in solvent A (water with 0.1% TFA). The fractions containing the correct peak were combined and lyophilised.

Analysis

HPLC Analysis: All purified peptides except Thr/Ser Analogue (**129**) were analysed using a using the same Dionex system as used for purification. An ACE 5 C18-AR analytical column (150 x 4.6 mm) was used with a flow rate of 1.0 mL min^{-1} and a linear gradient of 5-95% over 60 min (A = water, 0.1% TFA and B = acetonitrile, 0.1% TFA) and with UV detection at 214 and 254 nm unless otherwise stated. Thr/Ser Analogue (**129**) was analysed on an Agilent Technologies 1260 Infinity system using the same solvent system as above with detection at 215 nm.

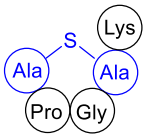
MS analysis: LRMS analysis was performed using the instrument described in Chapter 7.1. HRMS analysis for all peptides apart from ring B analogue (**138**) were performed on a Xevo G2-XS QTof with ESI source attached to a Water Acquity UPLC (Class I) system using LCMS grade water and acetonitrile both with 0.1% formic acid as an additive. Ring B analogue (**138**) was analysed using the Waters LCT Premier XE instrument as described in Chapter 7.1.

7.4 Experimental for Synthesis and Preparation of Ring AB Analogues of Nisin

Synthesis of Ring AB Analogues

All ring AB analogues were synthesised manually (Method 2, General Methods) using a pre-loaded Fmoc-Lys(Boc)-NovaSyn TGT resin (0.18 mmol g^{-1}).

Ring B Analogue (138)

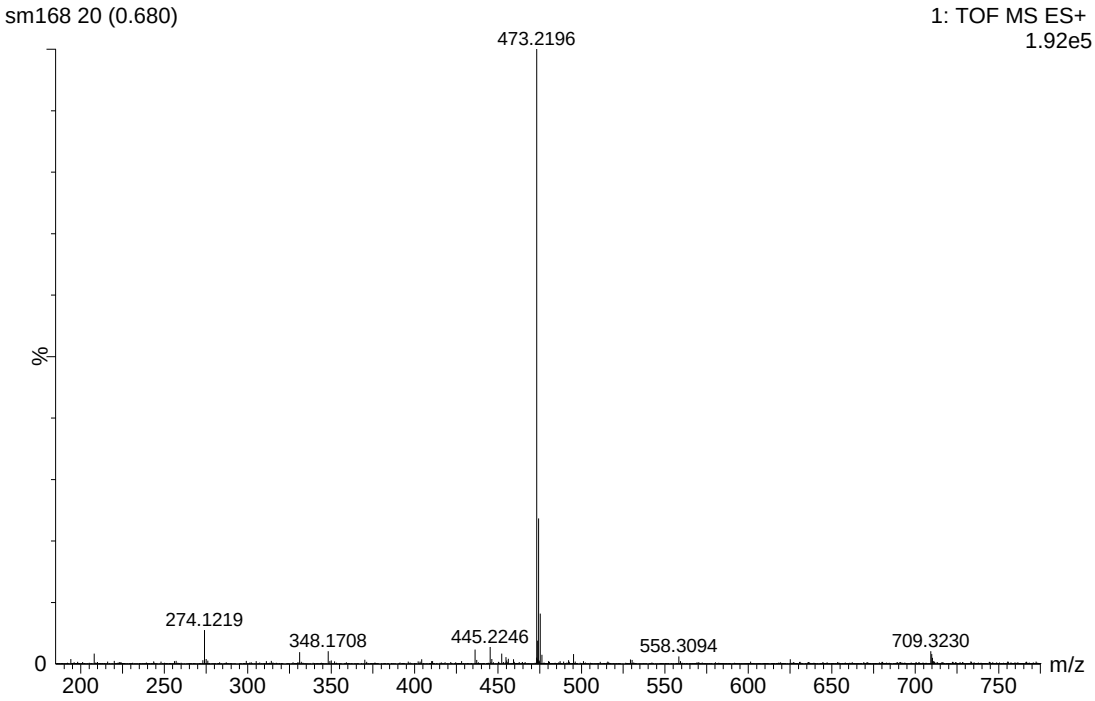


The resin (100 mg) was added to a reaction syringe, washed with DMF (4 x 1.5 mL) and left to swell for 20 min in 1.5 mL DMF. After this time, the Fmoc group was removed using the standard Fmoc deprotection step as described in General Methods above. Lanthionine (**60**) (36.4 mg, 3 eq.), PyAOP (46.9 mg, 5 eq.), HOAt (12.3 mg, 5 eq.) and DIPEA (31.4 µL, 10 eq.) were dissolved in DMF and added to the reaction syringe. The coupling proceeded as described in General Methods. Subsequent Fmoc deprotections and amino acid couplings were carried out as described in General Methods using the amounts listed below. Double couplings were used for all amino acid couplings except lanthionine for which microwave irradiation was used.

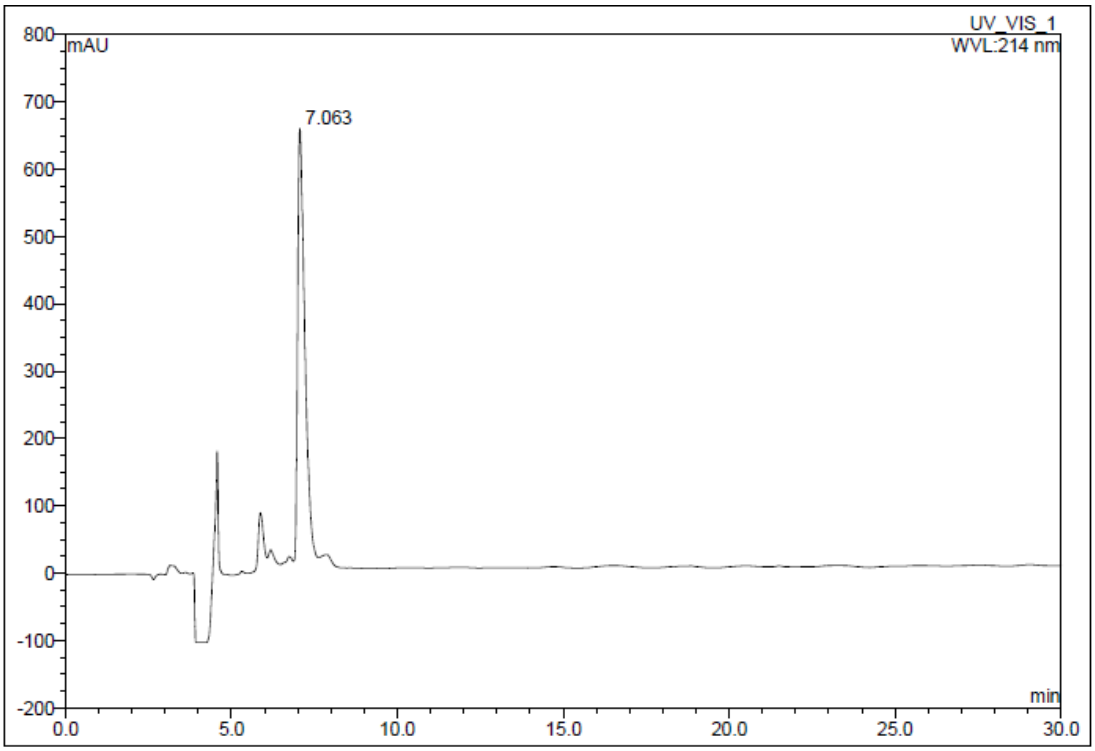
Amino Acid	Mass (mg)
Lanthionine (60)	36.4
Fmoc-Gly-OH	26.8 x 2
Fmoc-Pro-OH	30.4 x 2

The peptide was cleaved under standard conditions, washed with ether and lyophilised as described in General Methods. The peptide was first purified using a gradient of 5-40% over 30 mins at 2 mL min^{-1} using a Dr Maisch, Reprosil Gold 200 C8 semi-preparative column. A second purification was carried out using the same conditions to give purified (**138**), 200 µg, 5% yield. The purified peptide was analysed as outlined in General Methods.

HRMS: (ES$^+$) $[C_{19}H_{33}N_6O_6S]^+$ calculated = 473.2177; found *m/z* = 473.2196 $[M+H]^+$



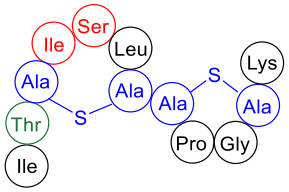
Analytical HPLC: flow rate of 1.0 mL min^{-1} and a linear gradient of 5-47.5% over 30 min using an Reprosil Gold 200 C8, 5 µm, 250 x 4.6 mm analytical column. R_t: 7.06 min.



General Procedure for the Synthesis of Amino Acid Symmetrical Anhydrides

The required Fmoc protected amino acid was dissolved in anhydrous CH_2Cl_2. DCC (0.5 eq.) was added and the reaction left to stir at room temperature for 2 h. After this time, the reaction mixture was filtered and the solvent removed in vacuo. The symmetrical anhydride was used without further purification.

Thr/Ser Analogue (129)



Red indicates use of pseudoproline

Method 1 - No Nα-alkylated amino acids - Unsuccessful

The resin (50 mg) was added to a reaction syringe, washed with DMF (4 x 1.5 mL) and left to swell for 20 min in 1.5 mL DMF. After this time, the Fmoc group was removed using the standard Fmoc deprotection step as described in General Methods above. Lanthionine (**60**) (18.2 mg, 3 eq.), PyAOP (23.5 mg, 5 eq.), HOAt (6.13 mg, 5 eq.) and DIPEA (15.7 µL, 10 eq.) were dissolved in DMF and added to the reaction syringe. The coupling proceeded as described in General Methods. Subsequent Fmoc deprotections and amino acid couplings were carried out as described in General Methods using the amounts listed below. Double couplings were used for all amino acid couplings except lanthionine for which microwave irradiation was used.

Amino Acid	Mass (mg)
Lanthionine	18.2
Fmoc-Gly-OH	13.4 x 2
Fmoc-Pro-OH	15.2 x 2
Second Lanthionine	18.2
Fmoc-Leu-OH	15.9 x 2
Fmoc-Ser(^{t}Bu)-OH	17.3 x 2
Fmoc-Ile-OH	15.9 x 2
Fmoc-Thr(^{t}Bu)-OH	17.9 x 2
Fmoc-Ile-OH	15.9 x 2

The peptide was cleaved under standard conditions, washed with ether and lyophilised as described in General Methods. A peak for the target compound was not observed in the crude LCMS analysis.

Method 2 - Use of Hmb-Leu - Unsuccessful

The resin (50 mg) was added to a reaction syringe, washed with DMF (4 x 1.5 mL) and left to swell for 20 min in 1.5 mL DMF. After this time, the Fmoc group was removed using the standard Fmoc deprotection step as described in General Methods above. Lanthionine (**60**) (18.2 mg, 3 eq.), PyAOP (23.5 mg, 5 eq.), HOAt (6.13 mg, 5 eq.) and DIPEA (15.7 µL, 10 eq.) were dissolved in DMF and added to the reaction syringe. The coupling proceeded as described in General Methods. Subsequent Fmoc deprotections and amino acid couplings were carried out as described in General Methods using the amounts listed below. Double couplings were used for all amino acid couplings except lanthionine for which microwave irradiation was used.

Amino Acid	Mass (mg)
Lanthionine	18.2
Fmoc-Gly-OH	13.4 x 2
Fmoc-Pro-OH	15.2 x 2
Second Lanthionine	18.2
Fmoc-(Fmoc-Hmb)Leu-OH	32.0 x 2
(Fmoc-Ser(^{t}Bu)-O)$_2$	68.7 x 2
Fmoc-Ile-OH	15.9 x 2
Fmoc-Thr(^{t}Bu)-OH	17.9 x 2
Fmoc-Ile-OH	15.9 x 2

The peptide was cleaved under standard conditions, washed with ether and lyophilised as described in General Methods. A peak for the target compound was not observed in the crude LCMS analysis.

Method 3 - Use of pseudoproline - Successful

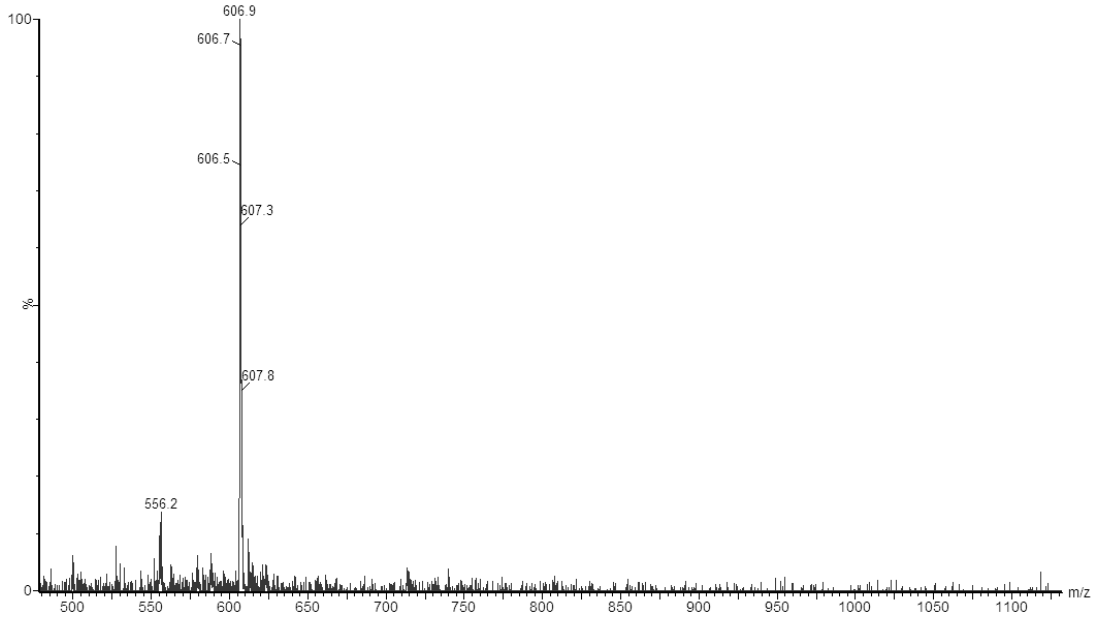
The resin (100 mg) was added to a reaction syringe, washed with DMF (4 x 1.5 mL) and left to swell for 20 min in 1.5 mL DMF. After this time, the Fmoc group was removed using the standard Fmoc deprotection step as described in General Methods above Lanthionine (**60**) (36.8 mg, 3 eq.), PyAOP (46.9 mg, 5 eq.), HOAt (12.3 mg, 5 eq.) and DIPEA (31.4 µL, 10 eq.) were dissolved in DMF and added to the reaction syringe. The coupling proceeded as described in General Methods. Subsequent Fmoc deprotections and amino acid couplings were carried out as described in General Methods using the amounts listed below. Double

couplings were used for all amino acid couplings except lanthionine for which microwave irradiation was used. The pseudoproline amino acid (43.3 mg, 5 eq.) was installed with standard amino acid coupling protocol.

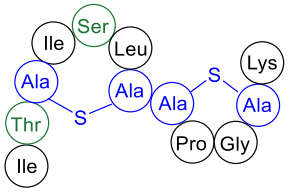
Amino Acid	Mass (mg)
Lanthionine	36.4
Fmoc-Gly-OH	26.8 x 2
Fmoc-Pro-OH	30.4 x 2
Second Lanthionine	36.4
Fmoc-Leu-OH	31.8 x 2
Fmoc-Ile-Ser[ψ(^{Me,Me})pro]-OH	43.3 x 2
Fmoc-Thr(^{t}Bu)-OH	35.8 x 2
Fmoc-Ile-OH	31.8 x 2

The peptide was cleaved under standard conditions, washed with ether and lyophilised as described in General Methods. The pseudoproline protected peptide (**158**) was carried forward crude.

LRMS: (ES^{+}) calculated $[C_{54}H_{92}N_{13}O_{15}S_2]^{+}$ = 1226.6272; found *m/z* = 606.9 $[M+2H]^{2+}$

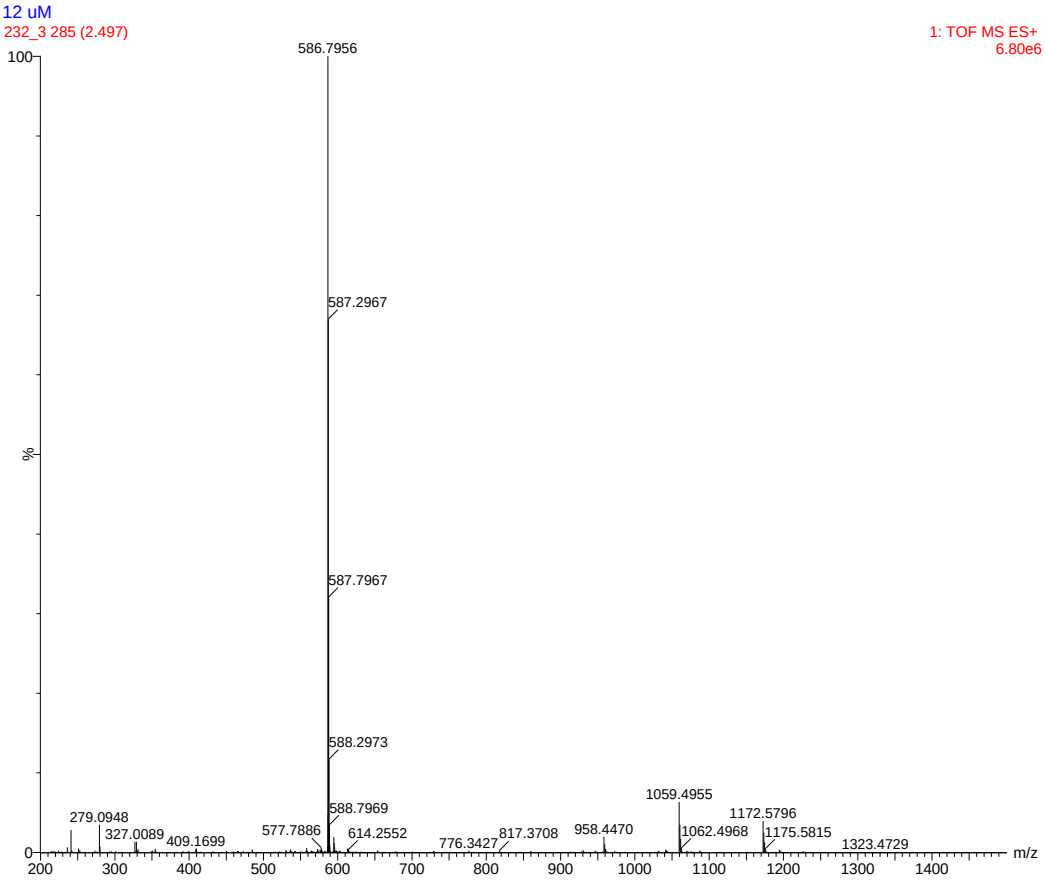


Pseudoproline Deprotection (129)

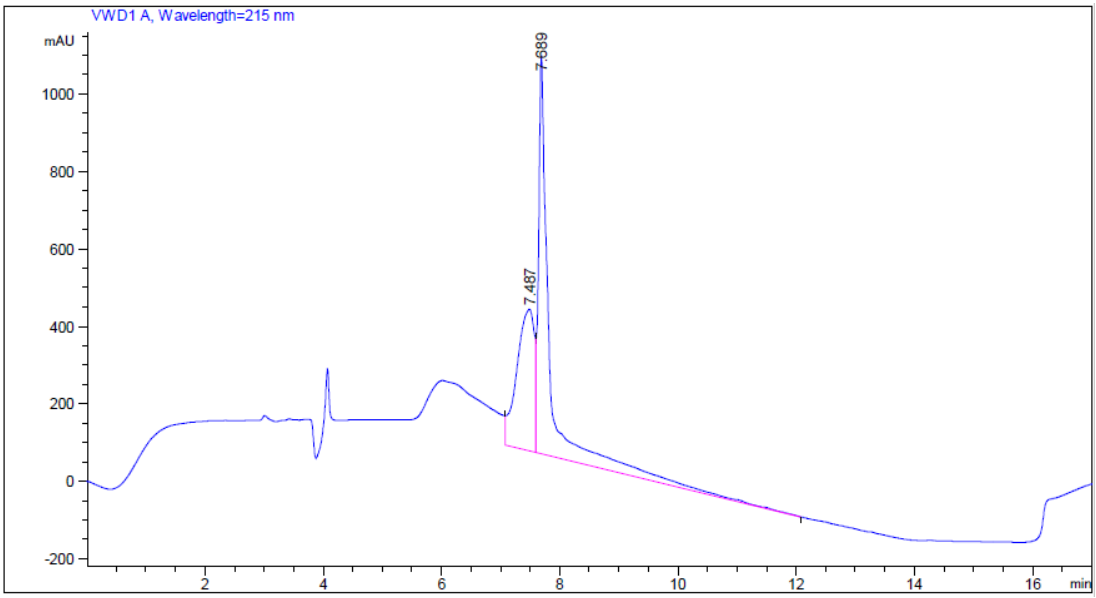


Crude AB analogue (**158**) (27 mg) was transferred to a 2 mL glass vial and cooled to 0 °C on ice. Triflic acid (300 µL) was added slowly. The vial was agitated every minute for 10 sec for a total of 5 min. The reaction mixture was added dropwise to a Falcon tube with dry ether (10 mL) at 0 °C. The Falcon tube was centrifuged for 10 min at 4000 rpm at 0 °C after which the diethyl ether was carefully decanted. This process was repeated with a further 2 aliquots of fresh diethyl ether (13 mL) after which the peptide was re-dissolved in water and lyophilised as described in General Methods. Purification took place using a gradient of 5-40% over 30 mins at 4 mL min^{-1} using a Phenomenex Onyx monolithic C18 column. Purification was repeated using the same conditions to give (**129**) 200 µg, 1% yield.

HRMS: (ES$^+$) $[C_{50}H_{86}N_{13}O_{15}S_2]^+$: 1172.5808; found *m/z* = 586.7956 $[M+2H]^{2+}$, 1172.5796 $[M+H]^+$

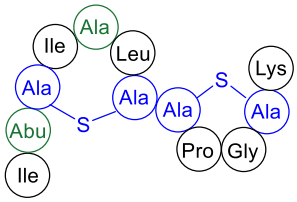


Analytical HPLC: flow rate of 1.0 mL min^{-1} and a linear gradient of 5-95% over 15 min using an Reprosil Gold 200 C8, 5 µm, 250 x 4.6 mm analytical column. R_t: 7.69 min.



Preliminary NMR work carried out by Dickman suggests this is one peptide.[204]

Abu/Ala Analogue (130)



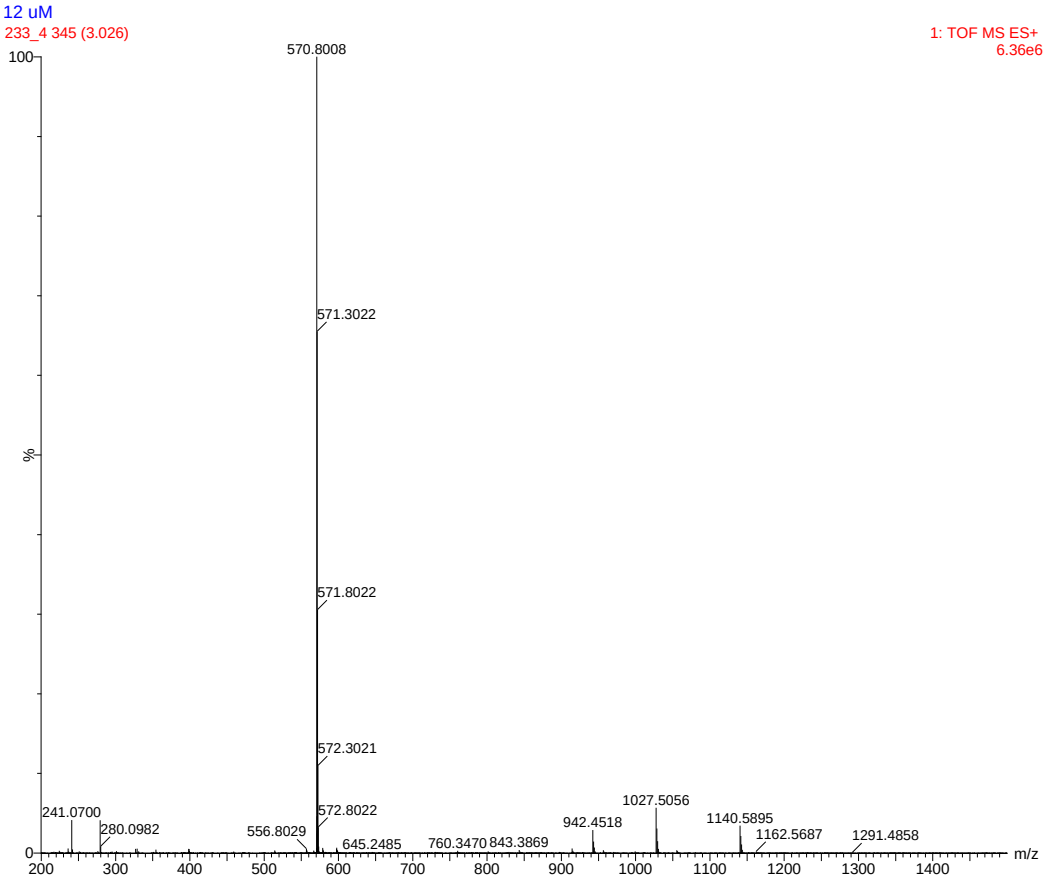
Method 1 - No Nα-alkylated amino acids - Successful

The resin (50 mg) was added to a reaction syringe, washed with DMF (4 x 1.5 mL) and left to swell for 20 min in 1.5 mL DMF. After this time, the Fmoc group was removed using the standard Fmoc deprotection step as described in General Methods above. Lanthionine (**60**) (18.2 mg, 3 eq.), PyAOP (23.5 mg, 5 eq.), HOAt (6.13 mg, 5 eq.) and DIPEA (15.7 µL, 10 eq.) were dissolved in DMF and added to the reaction syringe. The coupling proceeded as described in General Methods. Subsequent Fmoc deprotections and amino acid couplings were carried out as described in General Methods using the amounts listed below. Double couplings were used for all amino acid couplings except lanthionine for which microwave irradiation was used.

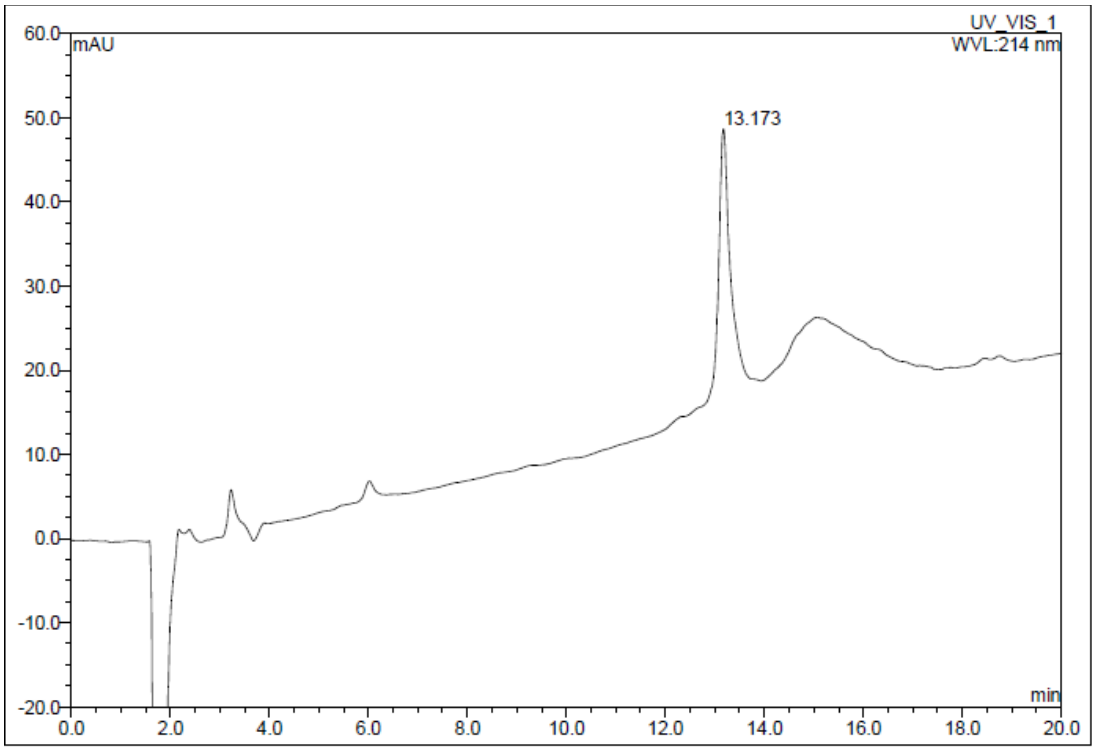
Amino Acid	Mass (mg)
Lanthionine	18.2
Fmoc-Gly-OH	13.4 x 2
Fmoc-Pro-OH	15.2 x 2
Second Lanthionine	18.2
Fmoc-Leu-OH	15.9 x 2
Fmoc-Ala-OH	14.0 x 2
Fmoc-Ile-OH	15.9 x 2
Fmoc-Abu-OH	14.6 x 2
Fmoc-Ile-OH	15.9 x 2

The peptide was cleaved under standard conditions, washed with ether and lyophilised as described in General Methods. The peptide was first purified using a gradient of 5-40% over 30 mins at 4 mL min^{-1} using a Phenomenex Onyx monolithic C18 column. A second purification was carried out using the same conditions to give (**130**) 300 µg, 1% yield.

HRMS: (ES^+) $[C_{50}H_{86}N_{13}O_{13}S_2]^+$: 1140.5909; found *m/z* = 570.8008 $[M+2H]^{2+}$, 1140.5895 $[M+H]^+$



Analytical HPLC: flow rate of 4.0 mL min^{-1} and a linear gradient of 5-20% over 20 min using a Phenomenex Onyx monolithic C18 column.



Method 2 – Use of Hmb-Ala - Unsuccessful

The resin (50 mg) was added to a reaction syringe, washed with DMF (4 x 1.5 mL) and left to swell for 20 min in 1.5 mL DMF. After this time, the Fmoc group was removed using the standard Fmoc deprotection step as described in General Methods above. Lanthionine (**60**) (18.2 mg, 3 eq.), PyAOP (23.5 mg, 5 eq.), HOAt (6.13 mg, 5 eq.) and DIPEA (15.7 µL, 10 eq.) were dissolved in DMF and added to the reaction syringe. The coupling proceeded as described in General Methods. Subsequent Fmoc deprotections and amino acid couplings were carried out as described in General Methods using the amounts listed below. Double couplings were used for all amino acid couplings except lanthionine for which microwave irradiation was used.

Amino Acid	Mass (mg)
Lanthionine	18.2
Fmoc-Gly-OH	13.4 x 2
Fmoc-Pro-OH	15.2 x 2
Second Lanthionine	18.2
Fmoc-Leu-OH	15.9 x 2
Fmoc-(Fmoc-Hmb)-Ala-OH	30.1 x 2
$(Fmoc\text{-}Ile\text{-}O)_2$	63.3 x 2
Fmoc-Abu-OH	14.6 x 2
Fmoc-Ile-OH	15.9 x 2

The peptide was cleaved under standard conditions, washed with ether and lyophilised as described in General Methods. A peak for the target compound was not observed in the crude LCMS analysis.

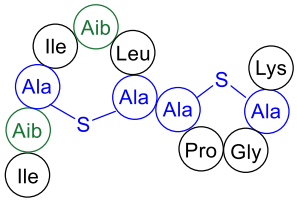
Method 3 - Use of Hmb-Leu - Unsuccessful

The resin (50 mg) was added to a reaction syringe, washed with DMF (4 x 1.5 mL) and left to swell for 20 min in 1.5 mL DMF. After this time, the Fmoc group was removed using the standard Fmoc deprotection step as described in General Methods above. Lanthionine (**60**) (18.2 mg, 3 eq.), PyAOP (23.5 mg, 5 eq.), HOAt (6.13 mg, 5 eq.) and DIPEA (15.7 µL, 10 eq.) were dissolved in DMF and added to the reaction syringe. The coupling proceeded as described in General Methods. Subsequent Fmoc deprotections and amino acid couplings were carried out as described in General Methods using the amounts listed below. Double couplings were used for all amino acid couplings except lanthionine for which microwave irradiation was used.

Amino Acid	Mass (mg)
Lanthionine	18.2
Fmoc-Gly-OH	13.4 x 2
Fmoc-Pro-OH	15.2 x 2
Second Lanthionine	18.2
Fmoc-(Fmoc-Hmb)-Leu-OH	32.0 x 2
(Fmoc-Ala-O)$_2$	55.7 x 2
Fmoc-Ile-OH	15.9 x 2
Fmoc-Abu-OH	14.6 x 2
Fmoc-Ile-OH	15.9 x 2

The peptide was cleaved under standard conditions, washed with ether and lyophilised as described in General Methods. A peak for the target compound was not observed in the crude LCMS analysis.

Aib Analogue (131)



The resin (50 mg) was added to a reaction syringe, washed with DMF (4 x 1.5 mL) and left to swell for 20 min in 1.5 mL DMF. After this time, the Fmoc group was removed using the standard Fmoc deprotection step as described in General Methods above. Lanthionine (**60**) (18.2 mg, 3 eq.), PyAOP (23.5 mg, 5 eq.), HOAt (6.13 mg, 5 eq.) and DIPEA (15.7 µL, 10 eq.) were dissolved in DMF and added to the reaction syringe. The coupling proceeded as described in General Methods. Subsequent Fmoc deprotections and amino acid couplings were carried out as described in General Methods using the amounts listed below. Double couplings were used for all amino acid couplings except lanthionine for which microwave irradiation was used.

Amino Acid	Mass (mg)
Lanthionine	18.2
Fmoc-Gly-OH	13.4 x 2
Fmoc-Pro-OH	15.2 x 2
Second Lanthionine	18.2
Fmoc-Leu-OH	15.9 x 2
Fmoc-Aib-OH	14.6 x 2
Fmoc-Ile-OH	15.9 x 2
Fmoc-Aib-OH	14.6 x 2
Fmoc-Ile-OH	15.9 x 2

The peptide was cleaved under standard conditions, washed with ether and lyophilised as described in General Methods. A peak for the target compound was not observed in the crude LCMS analysis.

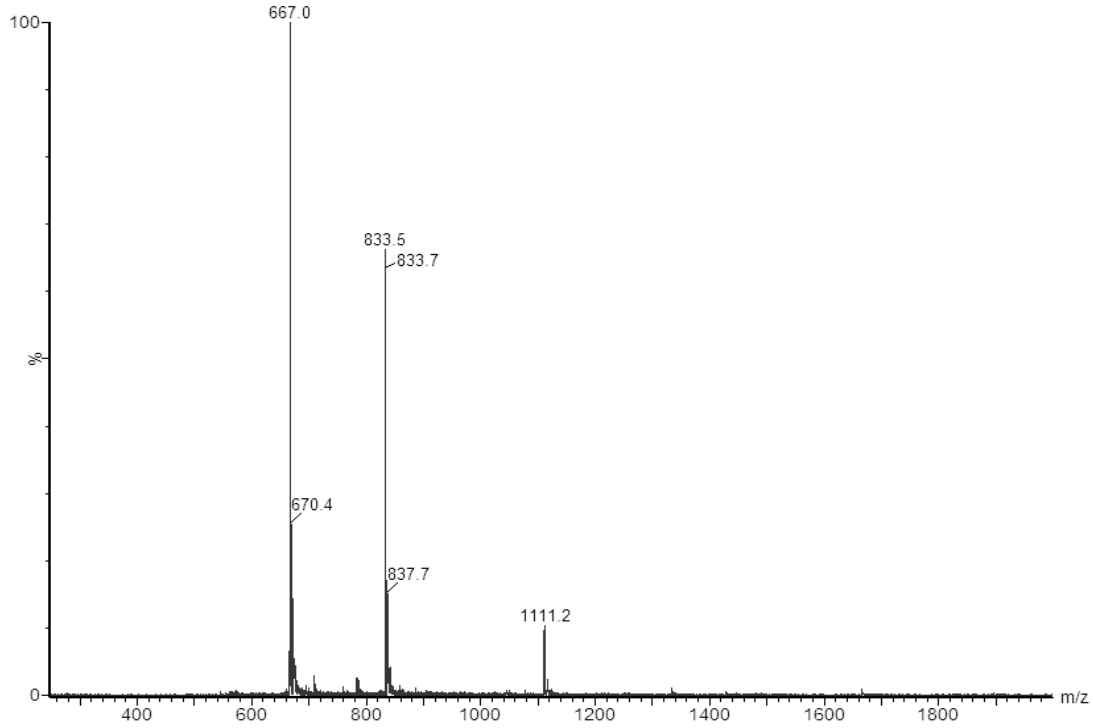
Recovery of Lanthionine (60)

The evacuated coupling solution was washed with a large excess of a solution of 5% citric acid and extracted with CH_2Cl_2. The resulting residue was purified by flash column chromatography (0-10% MeOH in CH_2Cl_2) to give clean lanthionine (**60**) 7.3 mg, 60% yield. Occasionally small amounts of DIPEA were still present after column chromatography and another washing with citric acid was required.

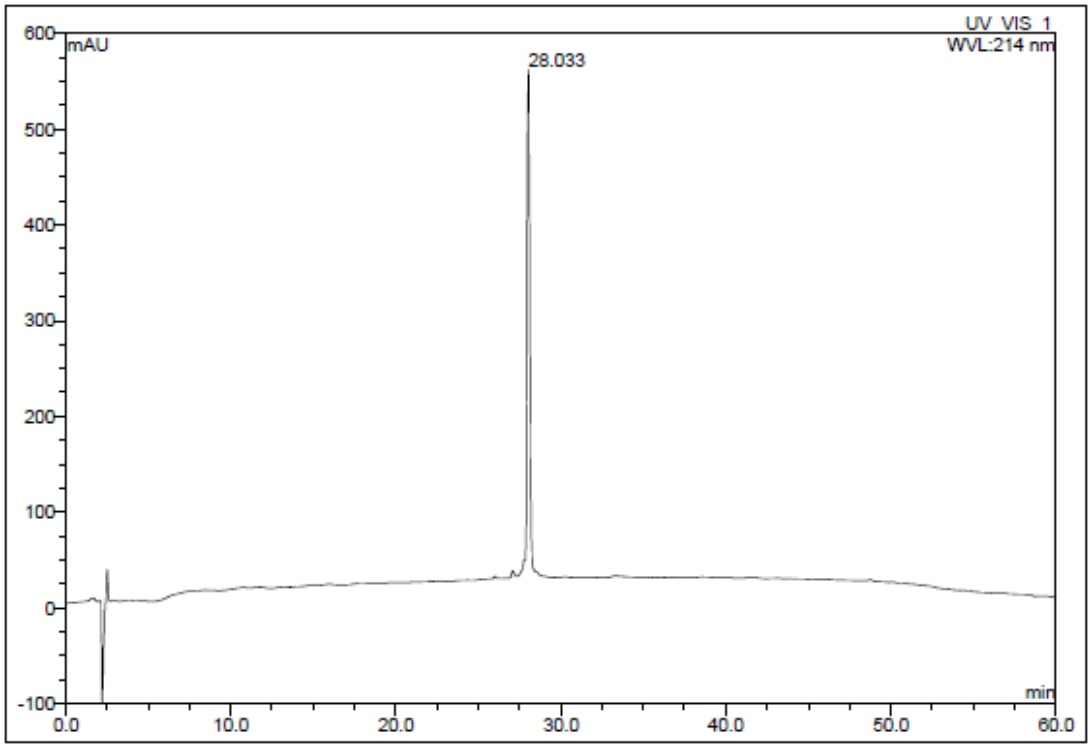
Enrichment of WT Nisin (27)

The commercially purchased nisin (25 g) was suspended in H_2O (600 mL) and stirred for 15 mins at room temperature. CH_2Cl_2 (500 mL) was added to give an emulsion which was transferred to falcon tubes and centrifuged for 15 min at 2500 rpm and 4 °C. A solid "pellet" was formed at the interface between H_2O and CH_2Cl_2 which was collected by filtration and dried at room temperature under reduced pressure. The solid was re-dissolved in H_2O (350 mL) and filtered through a bed of celite. The filtrate was concentrated *in vacuo* and lyophilised to give a fluffy brown solid (600 mg).

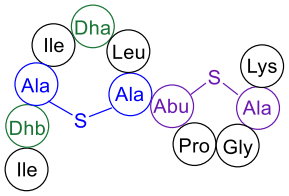
LRMS: (ES$^+$) $[C_{141}H_{229}O_{41}N_{38}S_7]^+$ calculated = 3334.5047; found *m/z* = 667.0 $[M+5H]^{5+}$, 833.5 $[M+4H]^{4+}$, 1111.2 $[M+3H]^{3+}$



Analytical HPLC: Standard conditions as described in General Methods. R_t: 28.03 min.

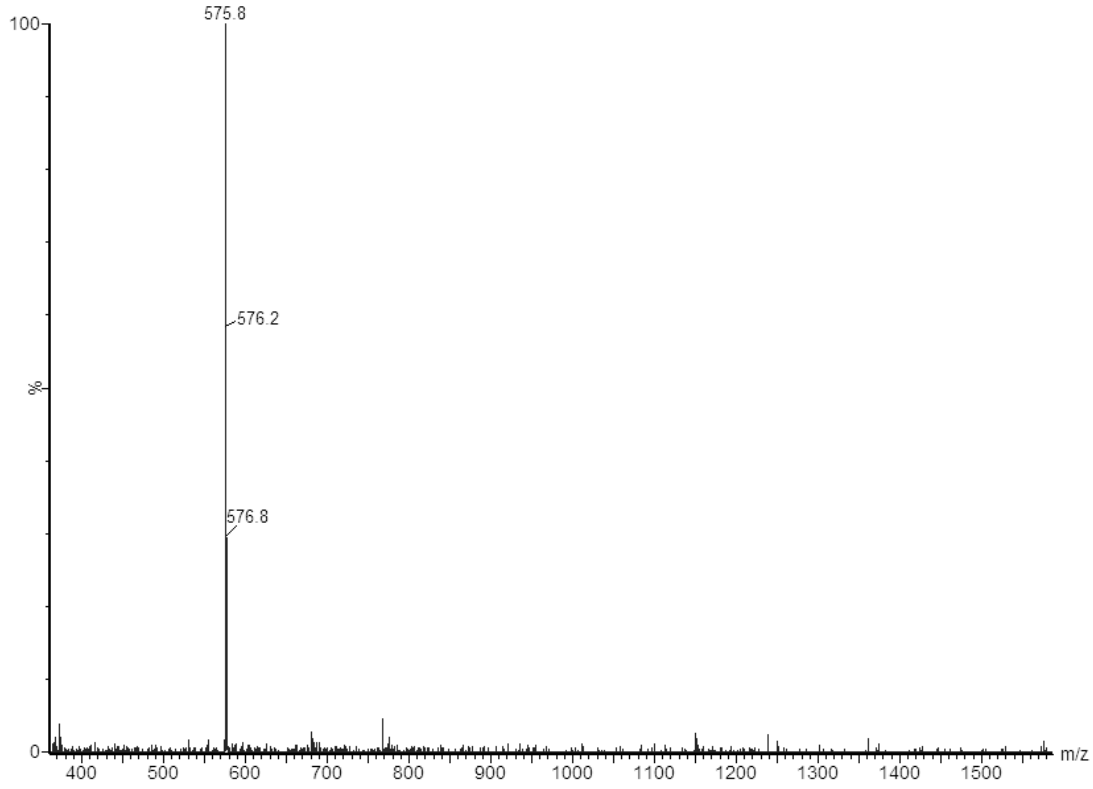


Digest of WT Nisin (93)[180]

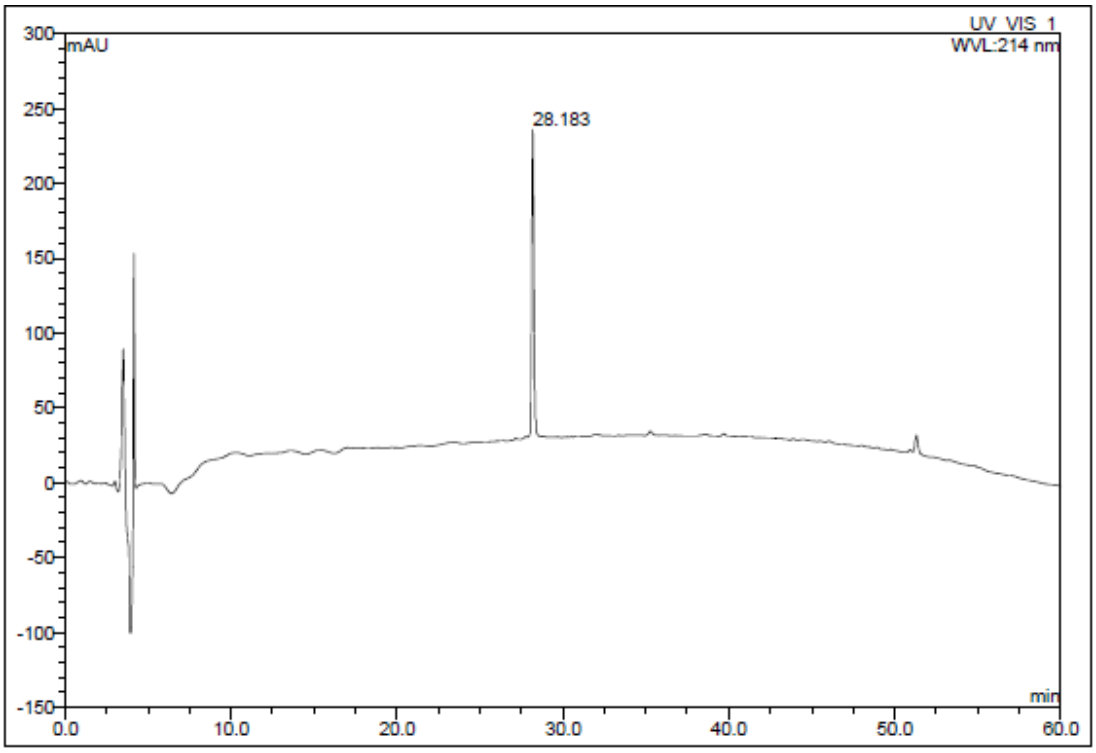


Enriched nisin (600 mg) was dissolved in 250 mL Tris acetate buffer (25 mmol NaOAc, 5 mmol Tris acetate, 5 mmol $CaCl_2$, adjusted to pH 7 with acetic acid). The solution was cooled on ice for 15 mins, trypsin (50 mg) was added and the reaction mixture was stirred at room temperature for 15 mins. After this time the reaction vessel was transferred to an incubator where it was agitated at 30 °C for 16 h. Another 50 mg of trypsin was added and the digestion was left for a further 24 h. After this time the reaction mixture was acidified with 1M HCl to a pH of 4 and then the reaction mixture was concentrated *in vacuo*. The resulting residue was lyophilised to give a brown fluffy powder. This was then purified directly using a gradient of 5-40% B using an ACE C8 150 x 10 mm semi-preparative column at 4 mL min^{-1} to give purified nisin (1-12) (**93**) (23.2 mg, 11%).

LRMS: (ES^+) [$C_{51}H_{84}N_{13}O_{13}S_2$] calculated = 1150.5753; found *m/z* = 575.8 $[M+2H]^{2+}$



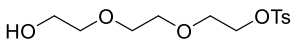
Analytical HPLC: Standard conditions as described in General Methods. R_t: 28.18 min.



7.5 Experimental for Hybrid Peptides

7.5.1 Hinge Region Linkers

Tetraethylene glycol *p*-toluenesulfonate (185)[236]



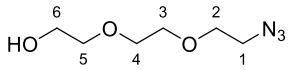
A solution of NaOH (800 mg, 20.0 mmol) in water (5 mL) was added to a reaction vessel containing triethylene glycol (**184**) (15.32 g, 102 mmol) dissolved in THF (10 mL). The reaction mixture was cooled to 0 °C. 4-Toluenesolfunyl chloride (2.4 g 12.6 mmol) in THF (16 mL) was added dropwise and the reaction mixture was left stirring at 0 °C for 2 h. After this time, the reaction mixture was poured onto ice and extracted with CH_2Cl_2 (2 x 200 mL). The combined organic layers were washed with water (2 x 300 mL) and then dried over $MgSO_4$ and concentrated *in vacuo* to give the title product (**185**) as a clear oil (3.12 g, 81% yield).

NMR: δ_H (600 MHz, $CDCl_3$) 2.46 (3H, s, C*H*$_3$), 3.58-3.59 (2H, m, C*H*$_2$), 3.62 (4H, s, 2 x C*H*$_2$), 3.70-3.73 (4H, m, 2 x C*H*$_2$), 4.17-4.19 (2H, m, C*H*$_2$OS), 7.35 (2H, d, *J* = 8.5, 2 x Ar), 7.81 (2H, d, *J* = 8.5, 2 x Ar).

NMR is in agreement with the literature[236]

LRMS: (ES^+) *m/z* = 305.4 $[M+H]^+$

2-(2-(2-Azidoethoxy)ethoxy)ethan-1-ol (186)[237]



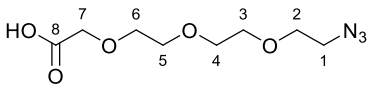
NaN_3 (1.42 g, 21.8 mmol) was added to a solution of tetraethylene glycol *p*-toluenesulfonate (**185**) (1.5 g, 4.93 mmol) in anhydrous DMF (50 mL). The reaction mixture was heated to 70 °C and left stirring overnight. After this time the solvent was removed *in vacuo* and the remaining residue was dissolved in CH_2Cl_2 and then washed with water (200 mL) and brine (200 mL). The organic layer was dried over $MgSO_4$ and concentrated *in vacuo* to give azide (**186**) as a clear oil (790 mg, 91% yield).

NMR: δ_H (600 MHz, $CDCl_3$) 3.40-3.42 (2H, m, C*H*$_2$N$_3$), 3.62-3.64 (2H, m, C*H*$_2$), 3.67-3.70 (6H, m, 3 x C*H*$_2$).

δ_c (150 MHz, $CDCl_3$) 50.6 (C1), 61.7 (C6), 70.0 (C2), 70.3 (C3), 70.6 (C5), 72.4 (C4)
NMR is in agreement with the literature[237]

LRMS: (ES^+) *m/z* = 351.2 $[2M+H]^+$

2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)acetic acid (187) [238]



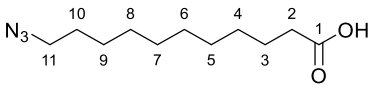
Azide (**186**) (348 mg, 1.98 mmol) was dissolved in THF (30 mL) and the reaction vessel was charged with Argon and cooled to 0 °C. A solution of NaH 60% in oil dispersion (317 mg, 7.93 mmol) in THF (5 mL) was added dropwise to the reaction mixture and it was left stirring for 40 minutes at 0 °C. After this time, a solution of bromoacetic acid (433 mg, 3.12 mmol) in THF (3 mL) was added dropwise to the reaction mixture and it was then left stirring at room temperature. After 3 h the THF was removed *in vacuo* and the reaction residue was dissolved in CH_2Cl_2 and water was added to quench any excess NaH. After 30 minutes of stirring at room temperature the aqueous layer was separated and acidified to

pH 2 using 1.0 M HCl. The aqueous phase was the extracted with CH_2Cl_2 (3 x 100 mL) and the combined organic layers were dried over $MgSO_4$ and concentrated *in vacuo* to give acid x as a clear oil to give acid (**187**) as a clear oil (247 mg, 53%).

NMR: δ_H (600 MHz, $CDCl_3$) 3.36-3.39 (2H, m, CH_2), 3.64-3.75 (10H, m, 5 x CH_2), 4.16 (2H, s, CH_2CO_2H) 8.99 (1H, bs, CO_2H).
NMR is in agreement with the literature.[238]

LRMS: (ES^-) *m/z* = 232.3 $[M-H]^-$

11-Azidoundecanoic acid (189)



NaN_3 (1.10 g 16.8 mmol) was added to a solution of 11-bromoundecanoic acid (1.00 g, 3.77 mmol) in DMF (20 mL) and the reaction mixture was left to stir overnight at room temperature. After this time, the reaction mixture was diluted with $KHSO_4$ (1M, 50 mL) and extracted with hexane (2 x 200 mL). The combined organic layers were dried over $MgSO_4$ and concentrated *in vacuo* to give acid (**189**) as a white, fluffy powder (651 mg, 76% yield).

NMR: δ_H (600 MHz, $CDCl_3$) 1.27-1.36 (12H, m, 6 x CH_2), 1.57-1.66 (4H, m, 2 x CH_2), 2.35 (2H, t, *J* = 7.5, CH_2CO_2H), 3.26 (2H, t, *J* = 7.0, CH_2N_3).

δ_c (150 MHz, $CDCl_3$) 24.6 (C3), 26.7 (C9), 28.8, 29.0, 29.1, 29.1, 29.3, 29.4, 34.0 (C (C2), 51.5 (C11), 180.1 (C1)
NMR is in agreement with the literature.[239]

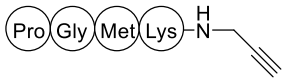
LRMS: (ES^-) *m/z* = 226.2 $[M-H]^-$

7.5.2 Hybrid Peptides

All SPPS for tail peptides and peptoids was carried out on Rink amide MBHA resin unless otherwise stated. Model peptides were synthesised manually and full length peptides were synthesised by automated peptide synthesis using protocols outlined in General Methods.

Model Compounds for Model Click Reactions

Model Propargylamine Coupling to Model Rings AB (198)



Preloaded Fmoc-Lys(Boc)-Wang resin (50 mg, 0.70 mmol g^{-1}) was added to a reaction syringe, washed with DMF (4 x 1.5 mL) and left to swell for 20 min in 1.5 mL DMF. After this time, the Fmoc group was removed using the standard Fmoc deprotection step as described in General Methods above. Subsequent amino acid couplings and Fmoc deprotections were carried out as described in General Methods using the amounts listed below. Double couplings were used for all amino acids.

Amino Acid	Mass (mg)
Fmoc-Met-OH	65.0
Fmoc-Gly-OH	52.0
Fmoc-Pro-OH	59.0

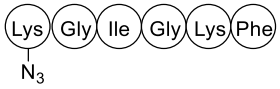
The peptide was cleaved under standard conditions, washed with ether and lyophilised as described in General Methods to give a crude yield of 15.0 mg.

LRMS: (ES^+) *m/z* = 432.5 $[M+H]^+$

Crude peptide (5.00 mg, 11.6 µmol) was dissolved in anhydrous DMF (50 µL) and propargylamine (37.0 µL, 580 µmol), BOP (10.0 mg, 23.2 µmol), and DIPEA (8.00 µL, 46.3 µmol) were added to the reaction mixture. This mixture was left agitating for 20 min after which time it was quenched with buffer (1 mL, 0.1% TFA in water), filtered and purified directly using reverse phase HPLC over a gradient of 5-60% at 3 mL min^{-1} using a Reprosil Gold 200 C8 semi-preparative column, *Rt* = 12.4 min, to give (**198**) (3.00 mg, 55% yield).

LRMS: (ES^+) *m/z* = 469.6 $[M+H]^+$

Truncated Pexiganan Analogue (199)



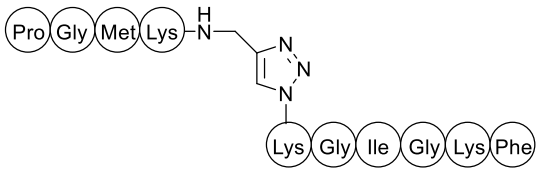
Preloaded Fmoc-Phe-Wang resin (50 mg, 0.61 mmol g^{-1}) was added to a reaction syringe, washed with DMF (4 x 1.5 mL) and left to swell for 20 min in 1.5 mL DMF. After this time, the Fmoc group was removed as described in General Methods above. Subsequent amino acid couplings and Fmoc deprotections were carried out as described in General Methods using the amounts listed below.

Amino Acid	Mass (mg)
Fmoc-Lys(Boc)-OH	71.5 x 2
Fmoc-Gly-OH	45.3 x 2
Fmoc-Ile-OH	53.9 x 2
Fmoc-Gly-OH	45.3 x 2
Fmoc-Lys(N_3)-OH	60.1 x 2

The peptide was cleaved under standard conditions, washed with ether and lyophilised as described in General Methods. (**183**) (15.2 mg, 74% yield). The peptide was used without further purification.

LRMS: (ES^+) *m/z* = 338.5 $[M+2H]^+$ 675.8 $[M+H]^+$

Model Click Chemistry (200)

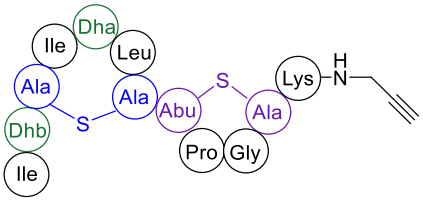


Alkyne (**198**) dissolved in 62.5 µL of DMF and azide (**183**) dissolved in 62.5 µL of water and added to a 250 µL microwave vial. Stock solutions of $CuSO_4.5H_2O$ (4.20 mg, 500 µL water) and NaAsc (6.70 mg, 500 µL water) were made up. From each of the stock solutions 50.0 µL was taken and added to the microwave vial. The reaction mixture was then irradiated for 20 min at 80 °C. After this time, the reaction mixture was quenched with buffer (3 mL, 0.1% TFA in water). The reaction mixture was assessed by HPLC using a gradient of 5-44% at 2

mL min^{-1} over 35 min using a Reprosil Gold 200 C8 semi-preparative column, R_t = 14.9 min, but the product (**200**) was not isolated.

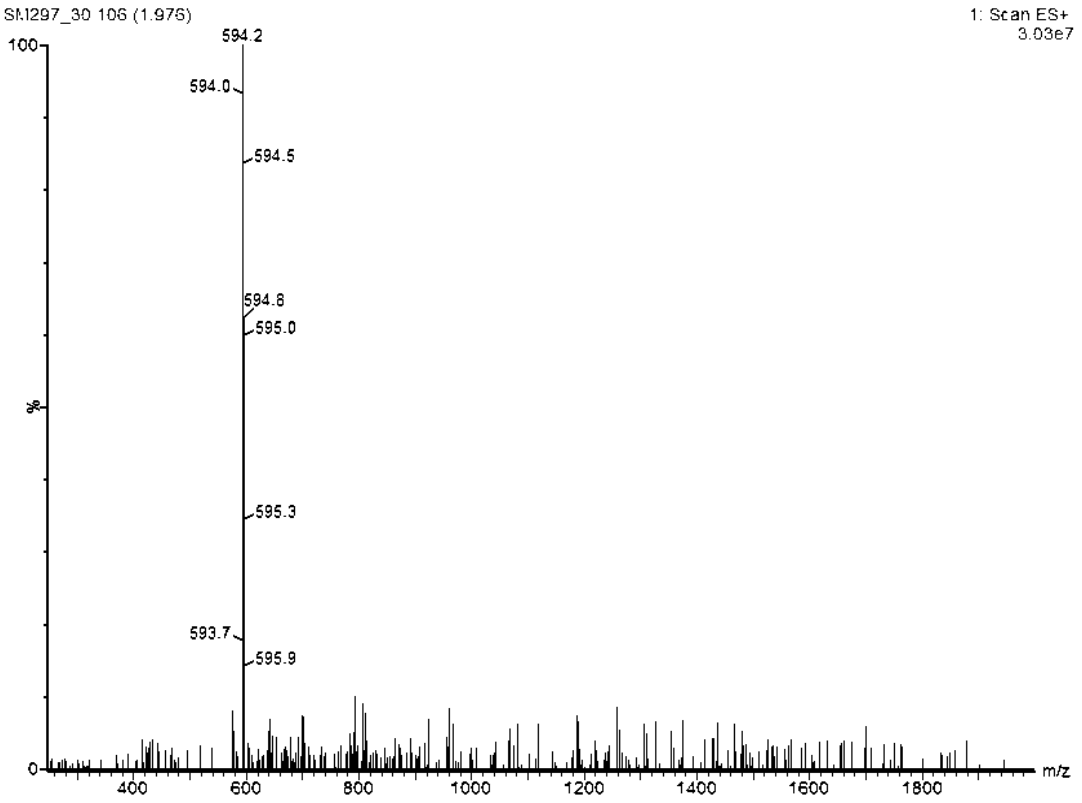
LRMS: (ES^+) *m/z* = 572.3 $[M+2H]^+$

Propargylamine Coupling to WT rings AB (110)[186]

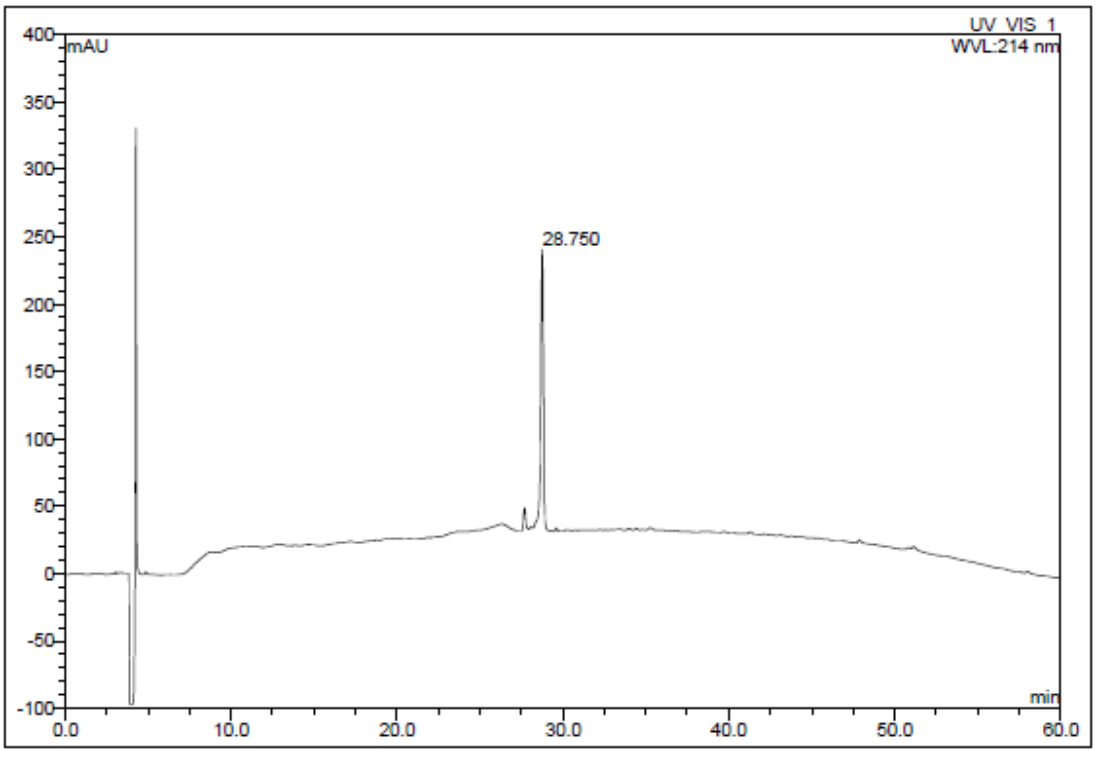


Purified WT rings AB (**93**) (5.00 mg, 4.35 µmol) was dissolved in anhydrous DMF (7.00 µL). Propargylamine (15.0 µL, 234 µmol) followed by BOP (4.00 mg, 9.04 µmol) and DIPEA (4.00 µL, 23.0 µmol) were added. The reaction mixture was left stirring for 20 min after which time it was quenched with buffer (3 mL, 1% TFA in water), filtered and purified directly. The crude mixture was purified using a gradient of 5-40% over 30 min at 4 mL min^{-1} using a C18 semi-preparative column to give alkylated rings AB (**110**) (3.10 mg, 61% yield).

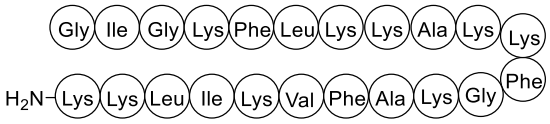
LRMS: (ES$^+$) $[C_{54}H_{87}N_{14}O_{12}S_2]^+$ calculated = 1187.6064; found *m/z* = 594.2 $[M+2H]^{2+}$



Analytical HPLC: Standard conditions as described in General Methods. R_t = 28.75 min.



Pexiganan (10)

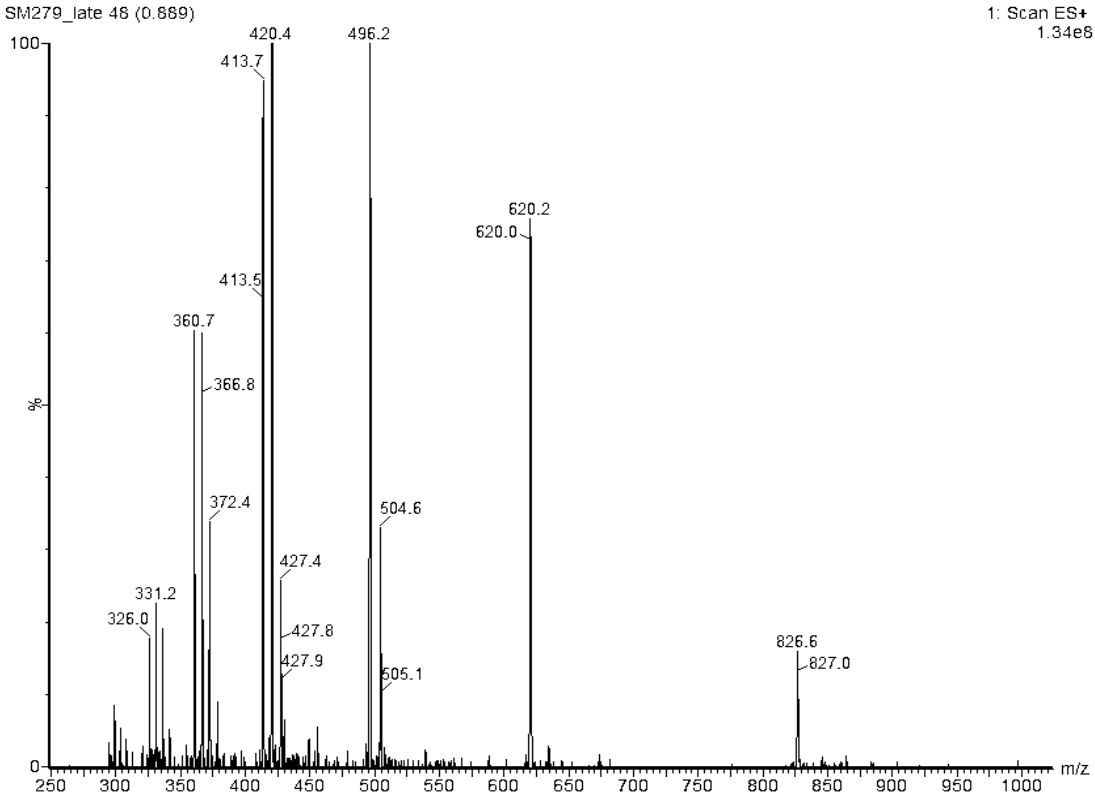


The Rink amide resin (50 mg, 0.7 mmol g^{-1}) was added to a reaction syringe, washed with DMF (4 x 1.5 mL) and left to swell for 20 min in 1.5 mL DMF. After this time, the Fmoc group was removed using the standard Fmoc deprotection step as described in General Methods above. Subsequent amino acid couplings and Fmoc deprotections were carried out as described in General Methods with stock solutions of amino acids as listed below.

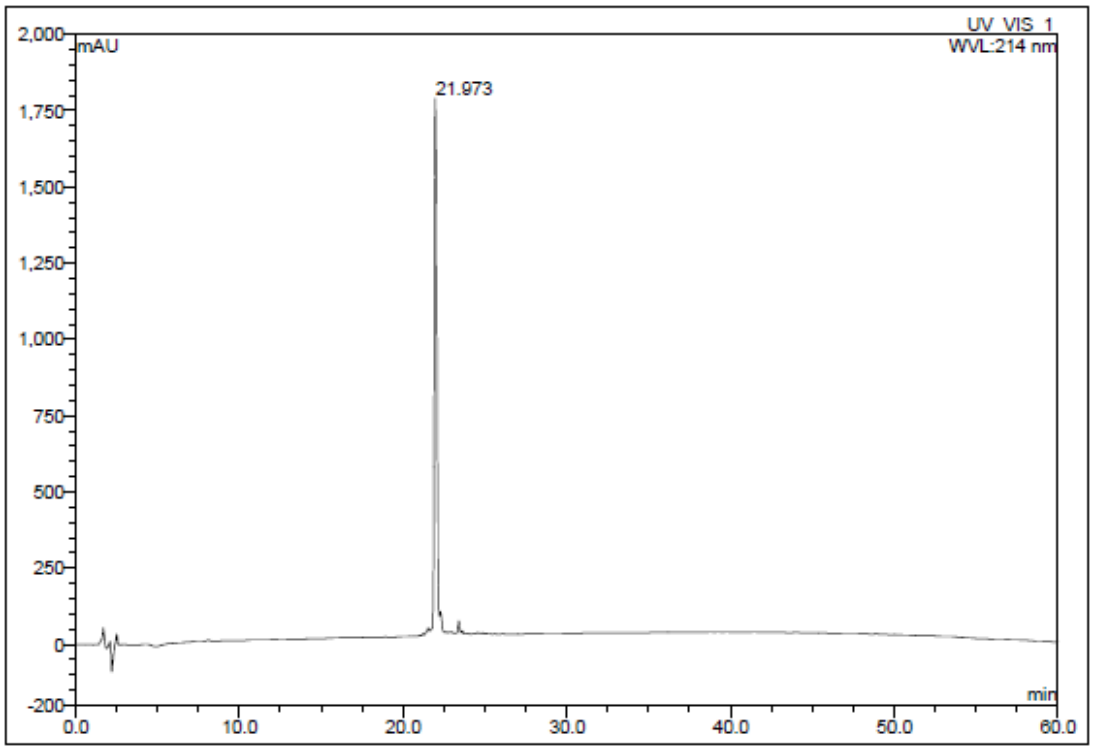
Amino Acid	Mass (mg)	Vol. DMF (mL)
Fmoc-Ala-OH	120	1.24
Fmoc-Phe-OH	218	1.76
Fmoc-Gly-OH	168	1.82
Fmoc-Ile-OH	136	1.22
Fmoc-Lys(Boc)-OH	764	4.97
Fmoc-Leu-OH	136	1.22
Fmoc-Val-OH	71.0	0.655

The peptide was cleaved under standard conditions, washed with ether and lyophilised as described in General Methods. The peptide was purified using a gradient of 5-40% over 30 min at 4 mL min^{-1} using a C18 semi-preparative column. The residue was purified a second time using the same conditions to give pexiganan (**10**) (8.7 mg, 7%).

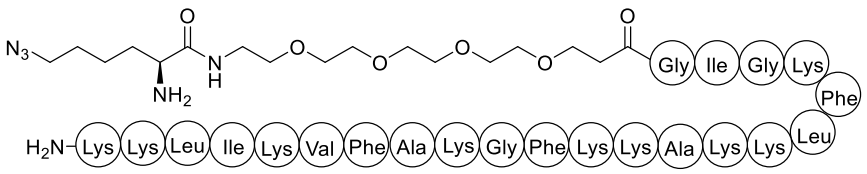
LRMS: (ES$^+$) calculated $[C_{122}H_{210}N_{32}O_{22}]^+$ = 2475.6292; found *m/z* = 826.6 $[M+3H]^{3+}$, 620.2 $[M+4H]^{4+}$, 496.2 $[M+5H]^{5+}$, 420.4 $[M+5H+K]^{6+}$, 413.7 $[M+6H]^{6+}$, 360.7 $[M+6H+K]^{7+}$



Analytical HPLC: Standard conditions as described in General Methods. R_t = 21.97 min.



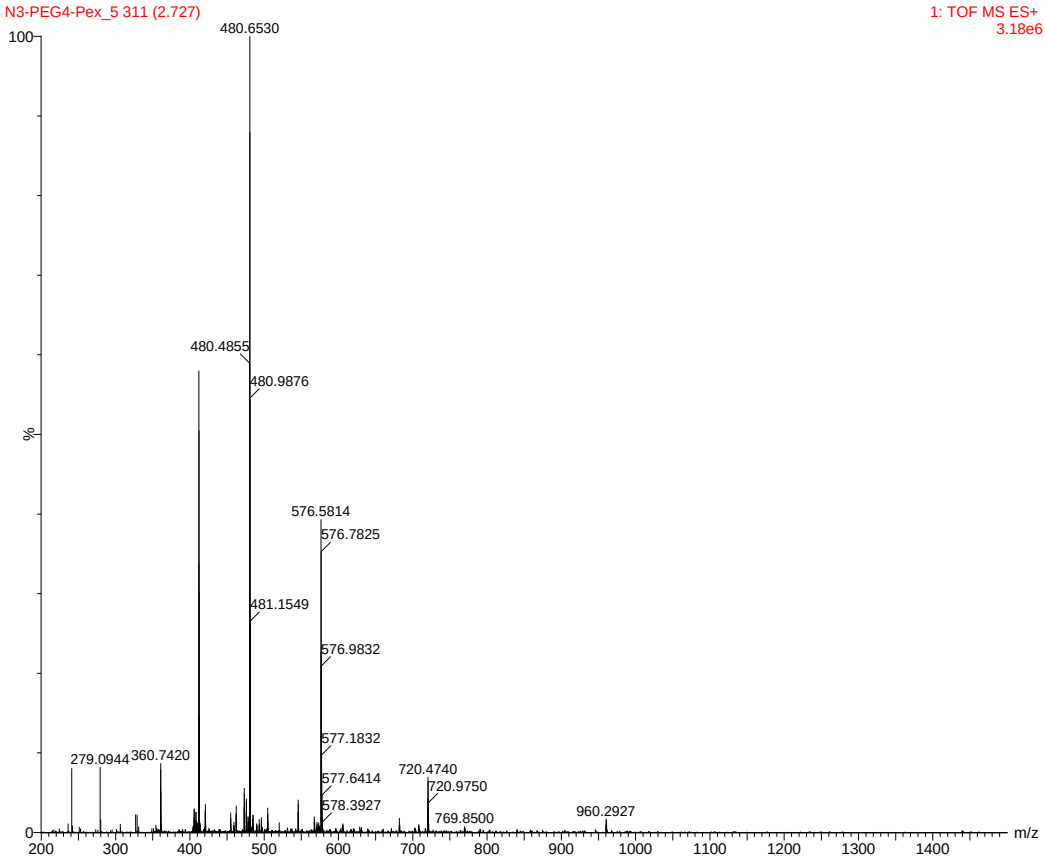
(N_3)Lys-PEG4-Pexiganan (183)



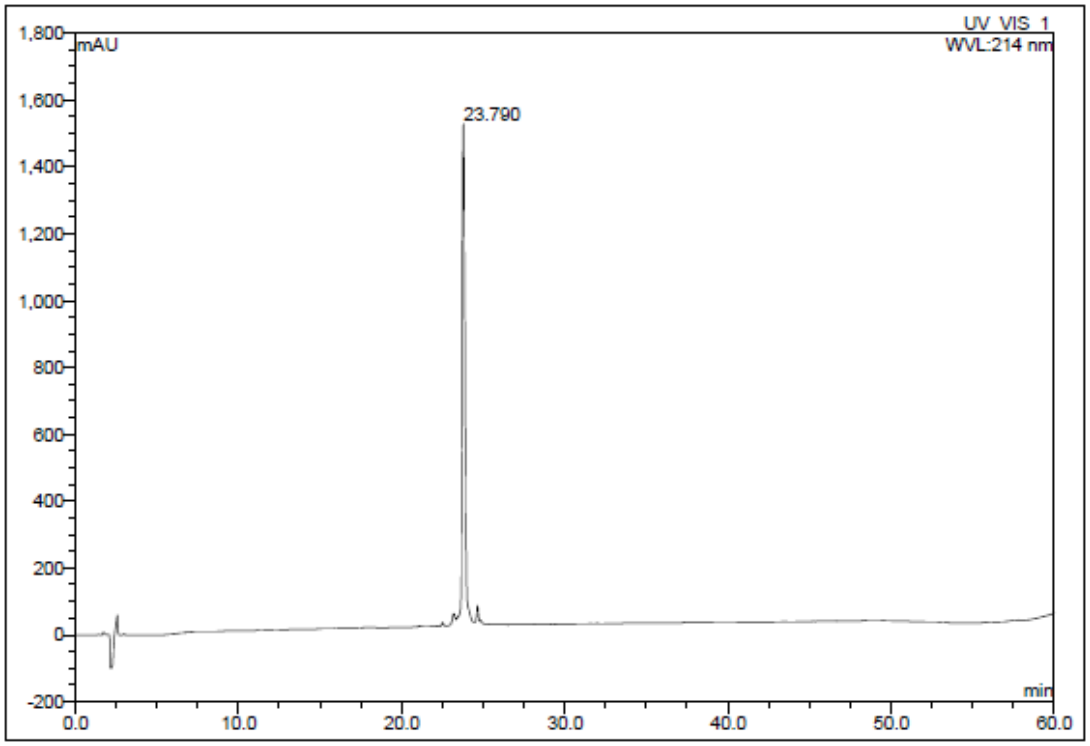
SPPS was carried out as described for pexiganan (**10**). Prior to cleavage Fmoc-PEG4-OH (**179**) was coupled using standard amino acid coupling protocol as described in General Methods. Fmoc deprotection was carried out as described in General Methods. Fmoc-Lys(N_3)-OH (**181**) was then coupled to the N-terminus using the standard amino acid coupling protocol and Fmoc deprotection was carried out as described in General Methods.

The peptide was cleaved under standard conditions, washed with ether and lyophilised as described in General Methods. The peptide was purified using a gradient of 5-40% over 30 min at 4 mL min^{-1} using a C18 semi-preparative column. The residue was purified a second time using the same conditions to give (**183**) (10.0 mg, 8%).

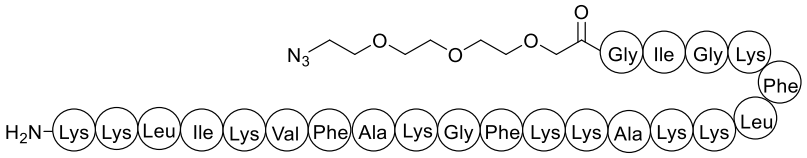
HRMS: (ES$^+$) calculated $[C_{139}H_{242}N_{37}O_{28}]^+$ = 2877.8645; found: *m/z* = 720.4740 $[M+4H]^{4+}$, 576.5814 $[M+5H]^{5+}$, 480.6530 $[M+6H]^{6+}$



Analytical HPLC: Standard conditions as described in General Methods. R_t = 23.79 min.



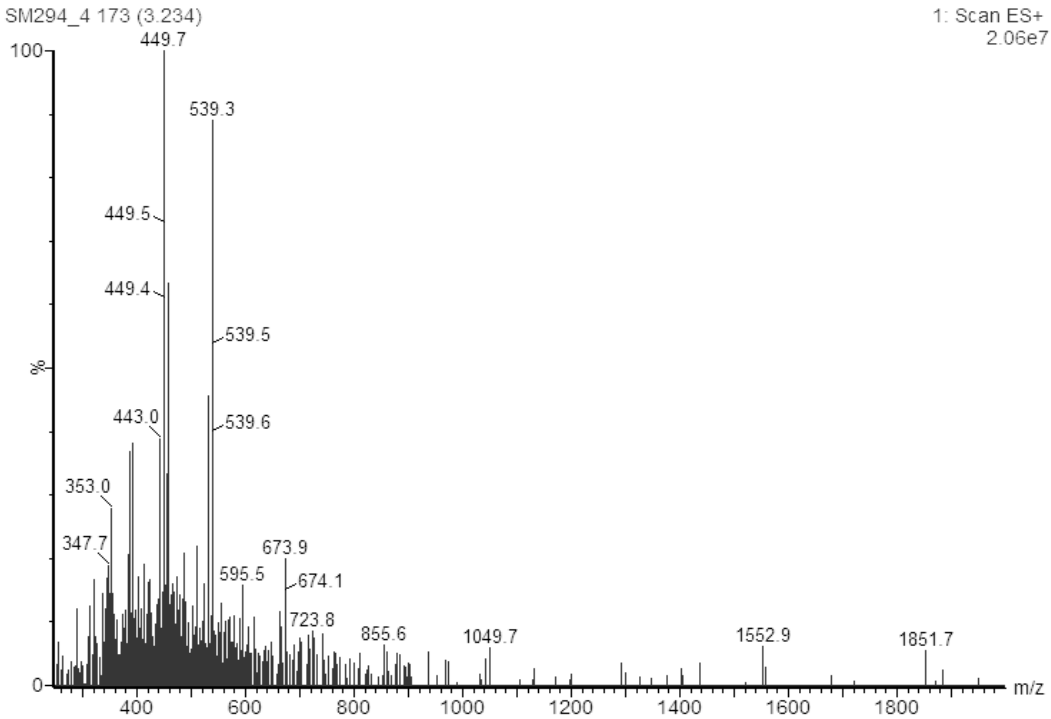
N_3-PEG3-Pexiganan (191)



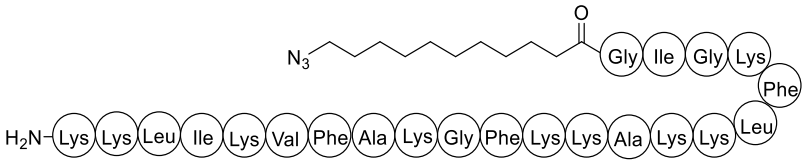
SPPS was carried out as described for pexiganan (**10**). Prior to cleavage N_3-PEG3-CO_2H (**187**) was coupled using standard amino acid coupling protocol as described in General Methods.

The peptide was cleaved under standard conditions, washed with ether and lyophilised as described in General Methods. The peptide was purified using a gradient of 5-45% over 30 min at 5 mL min^{-1} using a C18 semi-preparative column to give (**191**) (3.1 mg, 4%).

LRMS: (ES^+) calculated $C_{130}H_{223}N_{35}O_{26}$ = 2691.7276; found *m/z* = 674.1 $[M+4H]^{4+}$, 539.5 $[M+5H]^{5+}$, 449.8 $[M+6H]^{6+}$



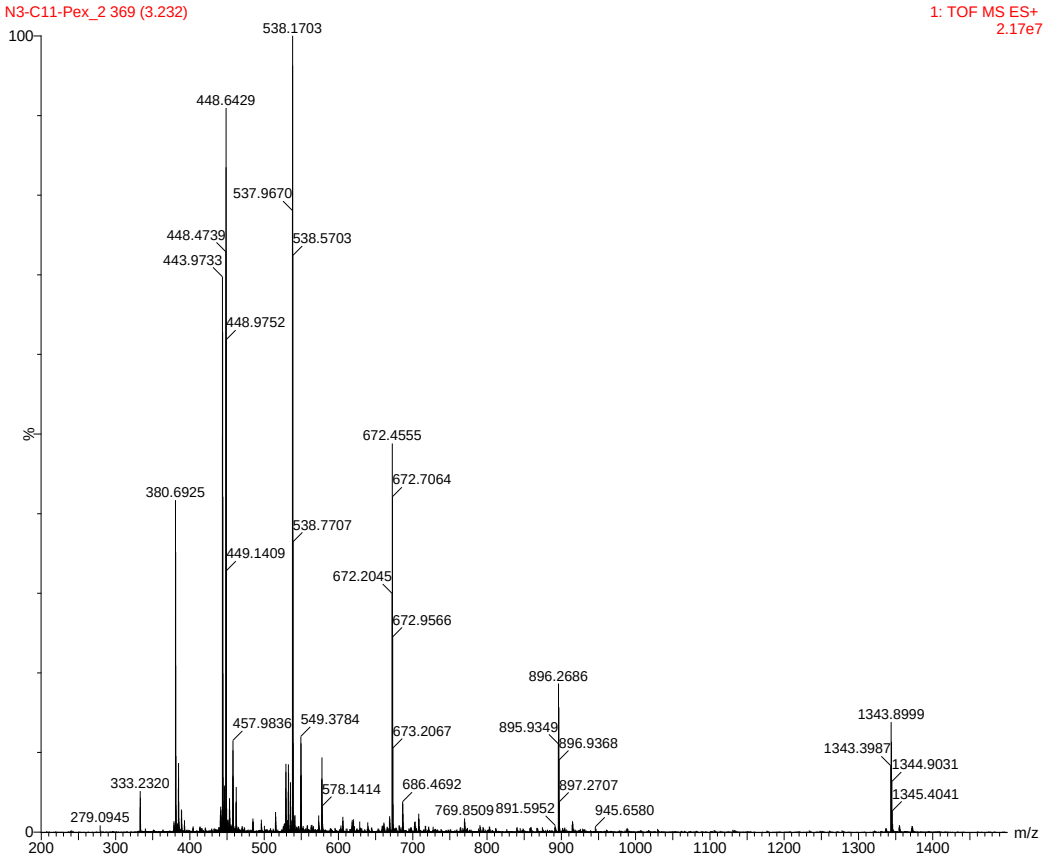
Pexiganan-C11-N_3 (193)



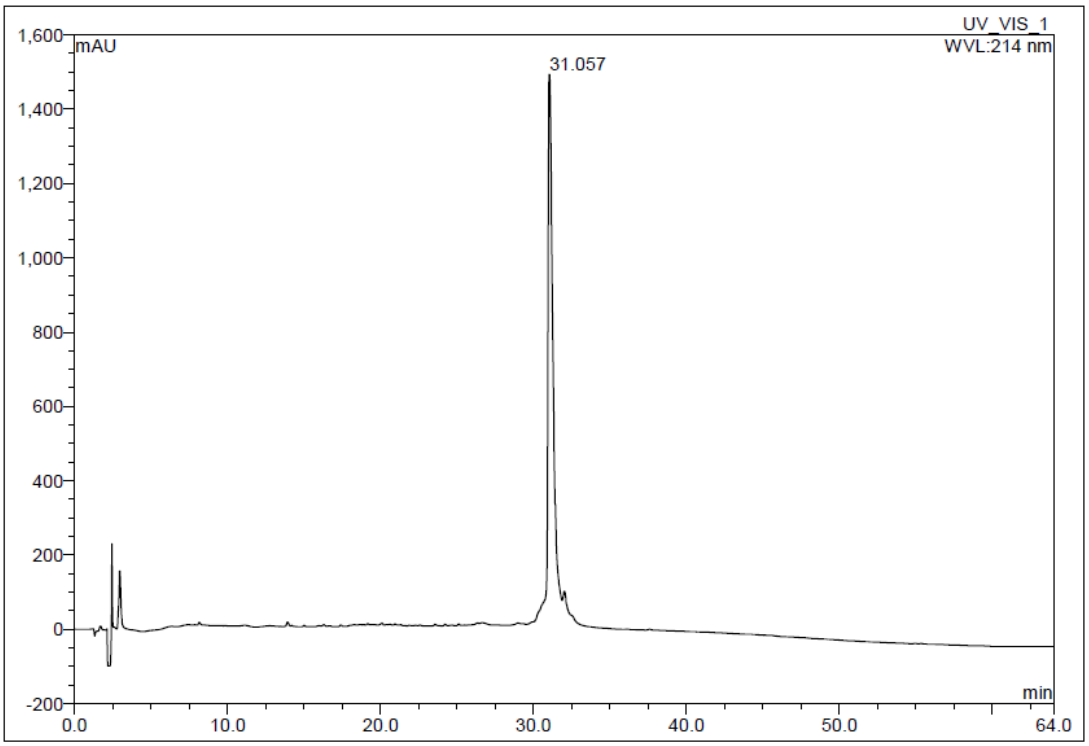
SPPS was carried out as described for pexiganan (**10**). Prior to cleavage 11-azidoundecanoic acid (**189**) was coupled using standard amino acid coupling protocol as described in General Methods.

The peptide was cleaved under standard conditions, washed with ether and lyophilised as described in General Methods. The peptide was purified using a gradient of 5-45% over 30 min at 5 mL min^{-1} using a C18 semi-preparative column to give (**193**) (2.90 mg, 3%).

HRMS: (ES^+) calculated $[C_{133}H_{229}N_{35}O_{23}]^+$ = 2685.7898; found: *m/z* = 1343.8999 $[M+2H]^{2+}$ 896.2686 $[M+3H]^{3+}$, 672.4555 $[M+4H]^{4+}$, 538.1703 $[M+5H]^{5+}$, 448.6429 $[M+6H]^{6+}$



Analytical HPLC: Standard conditions as described in General Methods. R_t = 31.06 min.



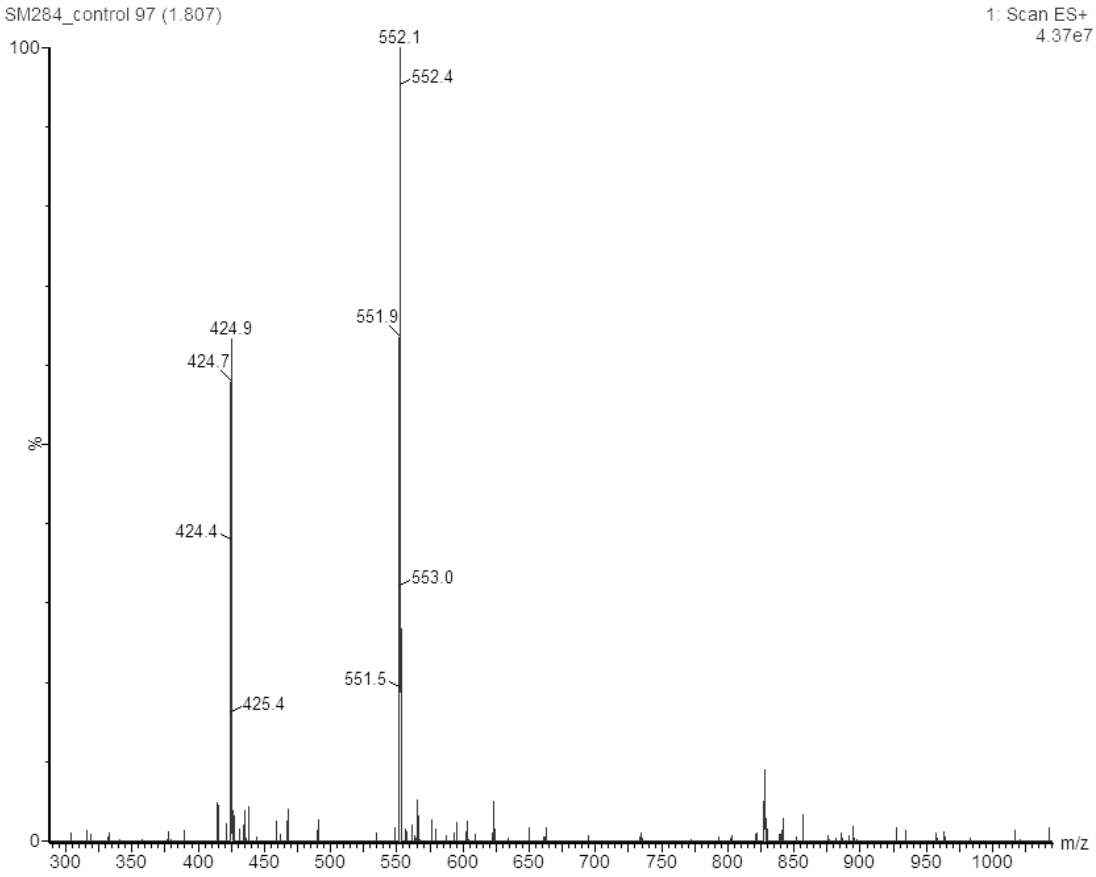
Mastoparan (12)

The Rink amide resin (50 mg, 0.7 mmol g^{-1}) was added to a reaction syringe, washed with DMF (4 x 1.5 mL) and left to swell for 20 min in 1.5 mL DMF. After this time, the Fmoc group was removed using the standard Fmoc deprotection step as described in General Methods above. Subsequent amino acid couplings and Fmoc deprotections were carried out as described in General Methods with stock solutions of amino acids as listed below. Double couplings were used for all amino acid couplings.

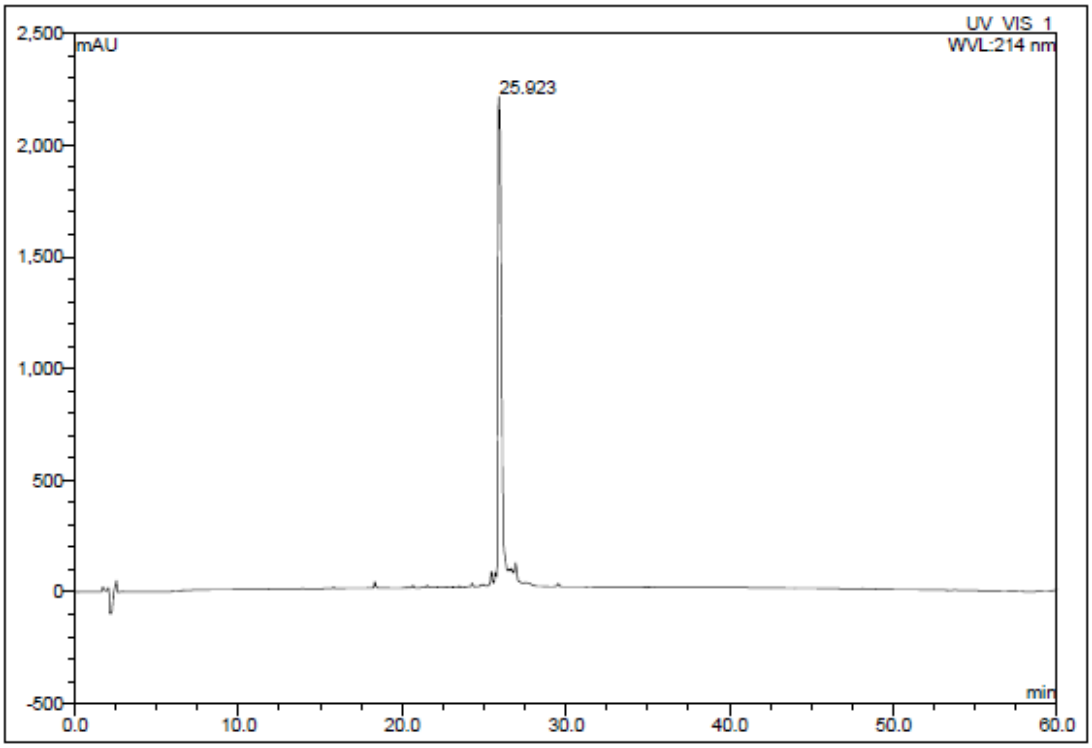
Amino Acid	Mass (mg)	Vol. DMF (mL)
Fmoc-Ala-OH	231	2.38
Fmoc-Asp(O^{t}Bu)-OH	205	2.31
Fmoc-Ile-OH	262	2.35
Fmoc-Lys-OH	514	3.35
Fmoc-Leu-OH	388	3.46
Fmoc-Gln-OH	236	1.16
Fmoc-Trp-OH	203	1.17

The peptide was cleaved under standard conditions, washed with ether and lyophilised as described in General Methods. The peptide was purified using a gradient of 5-40% over 30 min at 4 mL min^{-1} using a C18 semi-preparative column. The residue was purified a second time using the same conditions to give mastoparan (**12**) (4.1 mg, 5% yield).

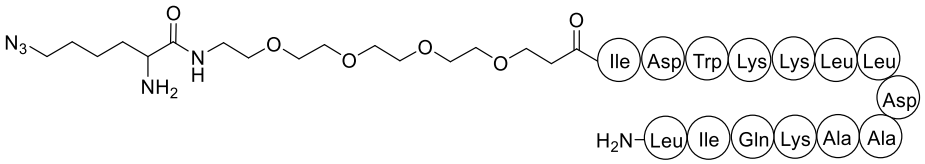
LRMS: (ES^+) [$C_{78}H_{133}N_{20}O_{19}$] calculated = 1654.0050; found *m/z* = 828.2 $[M+2H]^{2+}$, 552.2 $[M+3H]^{3+,}$ $[M+2Na+2H]^{4+}$



Analytical HPLC: Standard conditions as described in General Methods. R_t = 25.92 min.



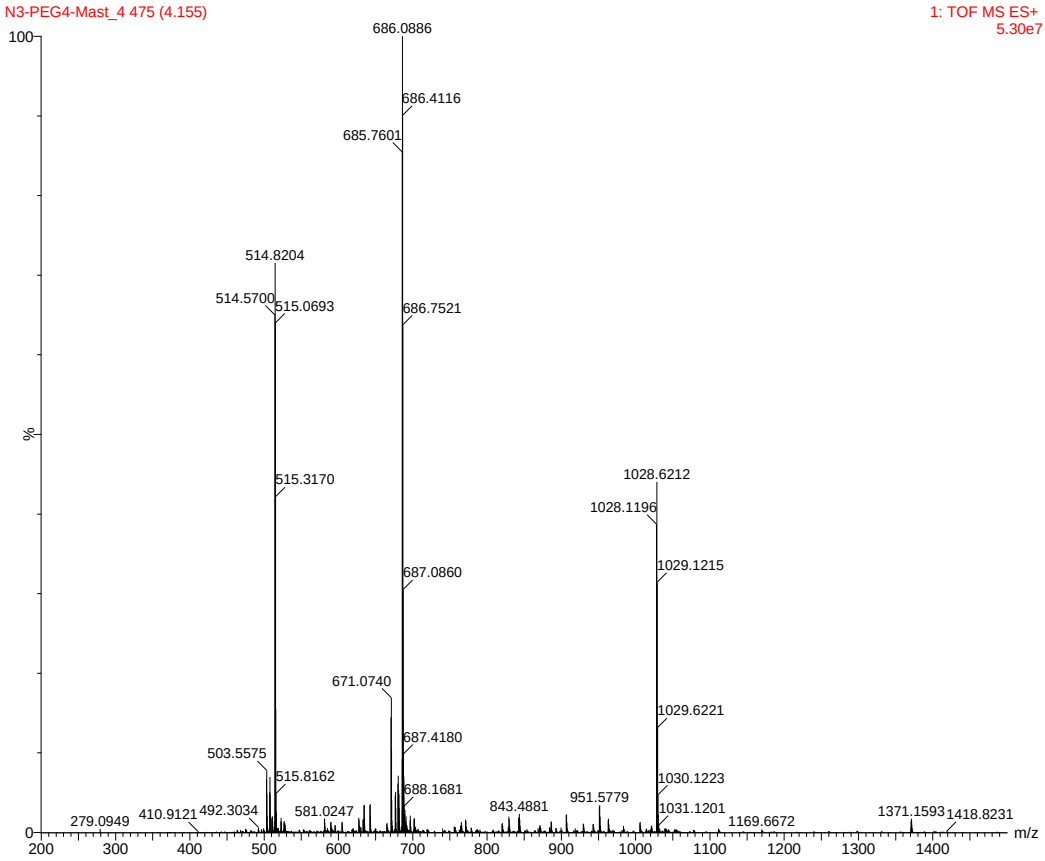
Lys(N_3)-PEG4-Mastoparan (190)



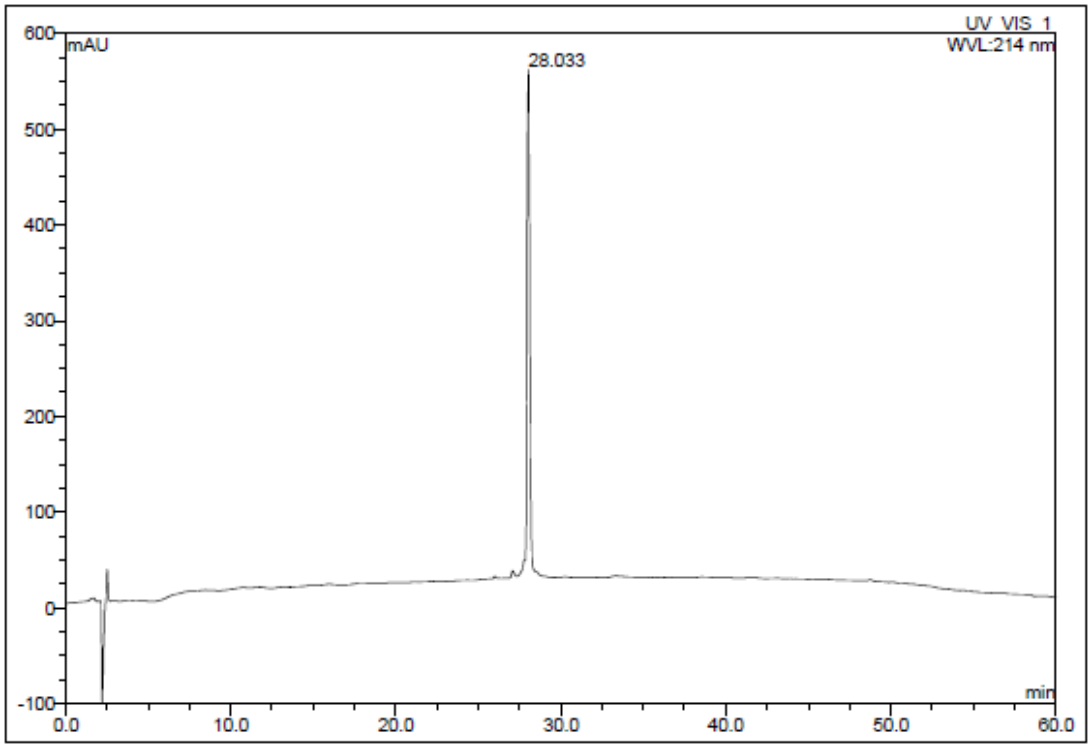
SPPS was carried out as described for mastoparan (**12**). Prior to cleavage Fmoc-PEG4-OH (**179**) was coupled using standard amino acid coupling protocol as described in General Methods. Fmoc deprotection was carried out as described in General Methods. Fmoc-Lys(N_3)-OH (**181**) was then coupled to the N-terminus using the standard amino acid coupling protocol and Fmoc deprotection was carried out as described in General Methods.

The peptide was cleaved under standard conditions, washed with ether and lyophilised as described in General Methods. The peptide was purified using a gradient of 5-40% over 30 min at 4 mL min^{-1} using a C18 semi-preparative column. The residue was purified a second time using the same conditions to give (**190**) (6.0 mg, 7%).

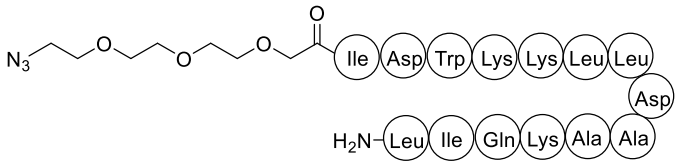
HRMS: (ES$^+$) $[C_{95}H_{164}N_{25}O_{25}]^+$ calculated = 2055.2325; found: *m/z* = 1028.6212 $[M+2H]^{2+}$, 686.0886 $[M+3H]^{3+}$, 514.8204 $[M+4H]^{4+}$



Analytical HPLC: Standard conditions as described in General Methods. R_t = 28.03 min.



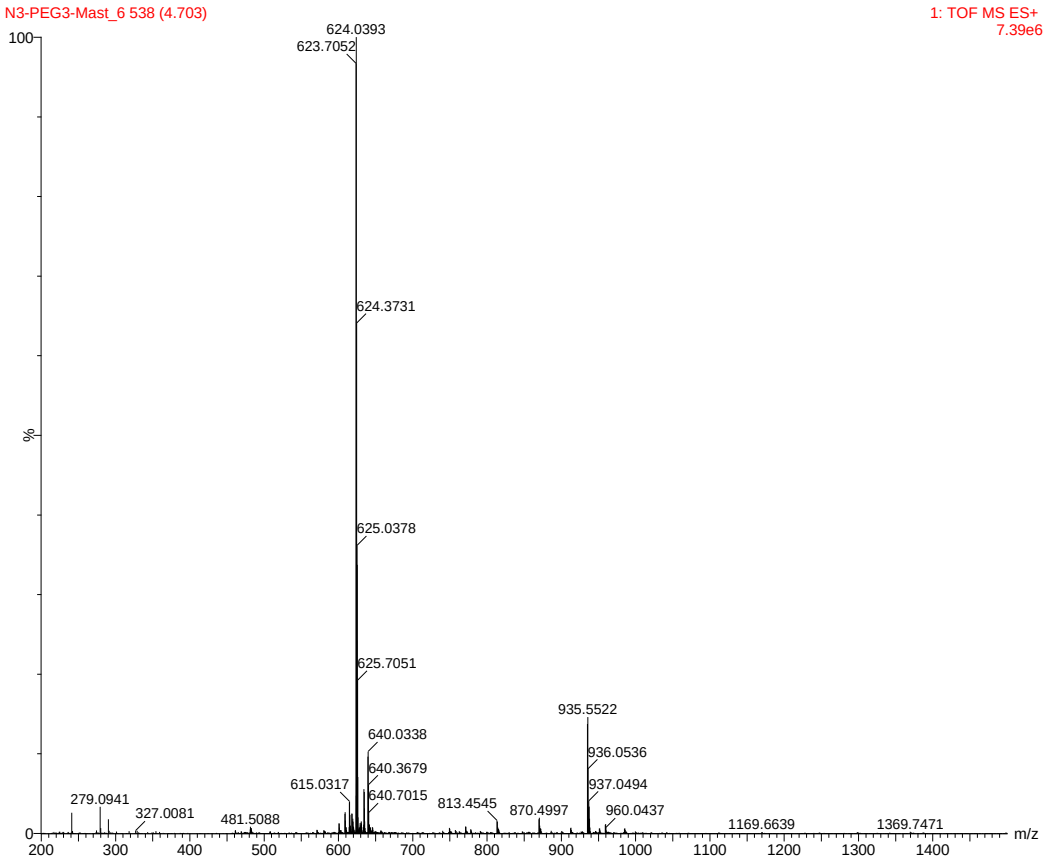
N_3-PEG3-Mastoparan (192)



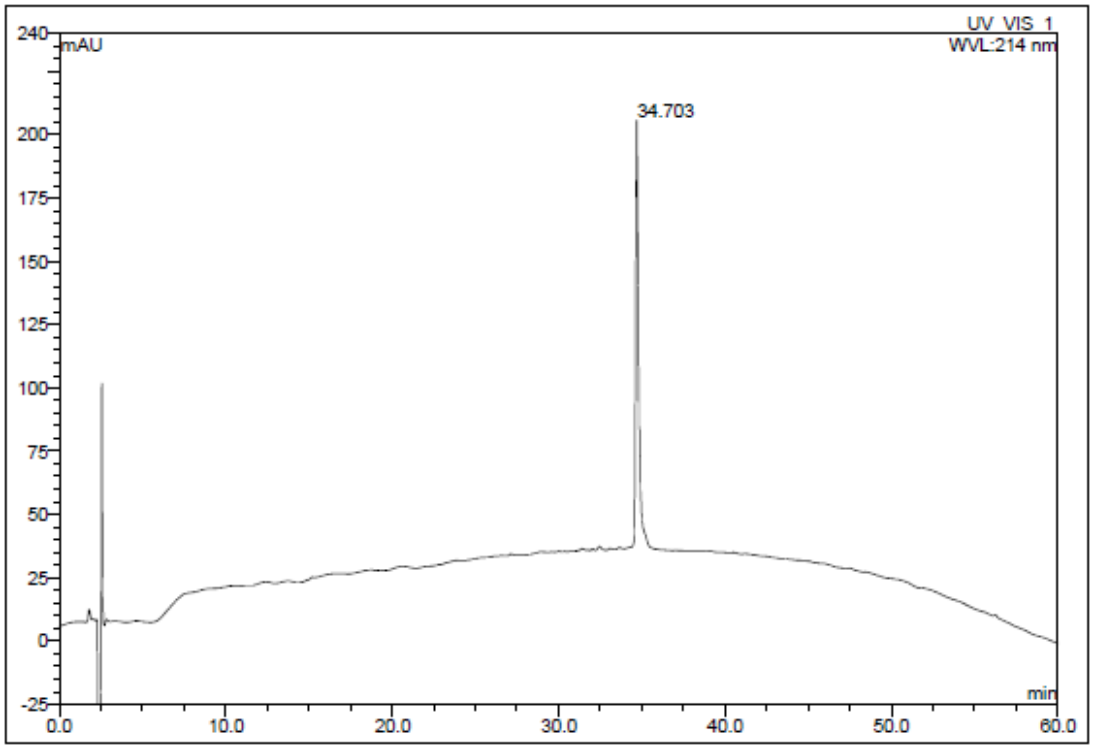
SPPS was carried out as described for mastoparan (**10**). Prior to cleavage N_3-PEG3-CO_2H (**187**) was coupled using standard amino acid coupling protocol as described in General Methods. Fmoc deprotection was carried out as described in General Methods.

The peptide was cleaved under standard conditions, washed with ether and lyophilised as described in General Methods. The peptide was purified using a gradient of 5-40% over 30 min at 4 mL min^{-1} using a C18 semi-preparative column. The residue was purified a second time using the same conditions to give (**192**) (8.0 mg, 9% yield).

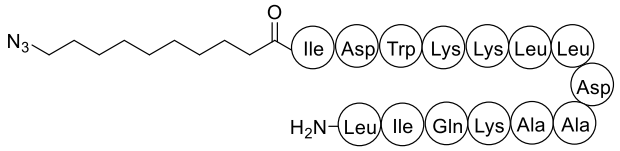
HRMS: (ES^+) $[C_{86}H_{146}N_{23}O_{23}]^+$ calculated = 1869.0956; found: *m/z* = 935.5522 $[M+2H]^{2+}$, 624.0393 $[M+3H]^{3+}$



Analytical HPLC: Standard conditions as described in General Methods. R_t = 34.70 min.



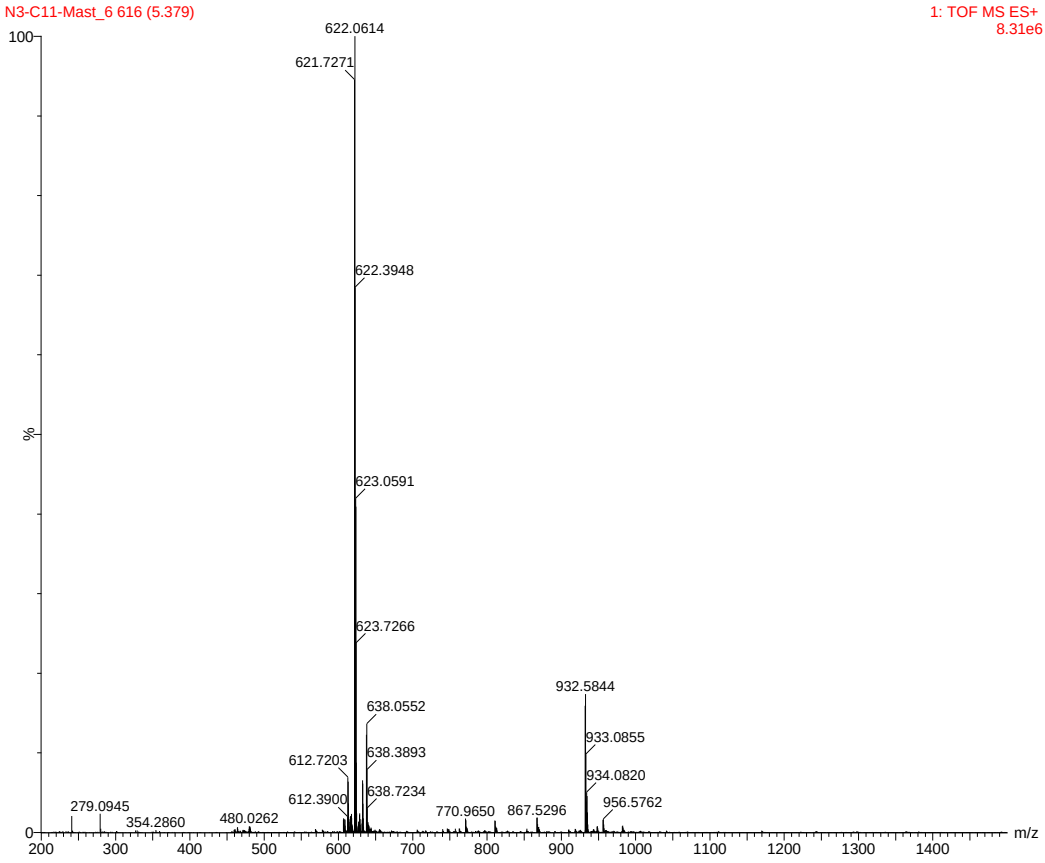
N_3-C11-Mastoparan (194)



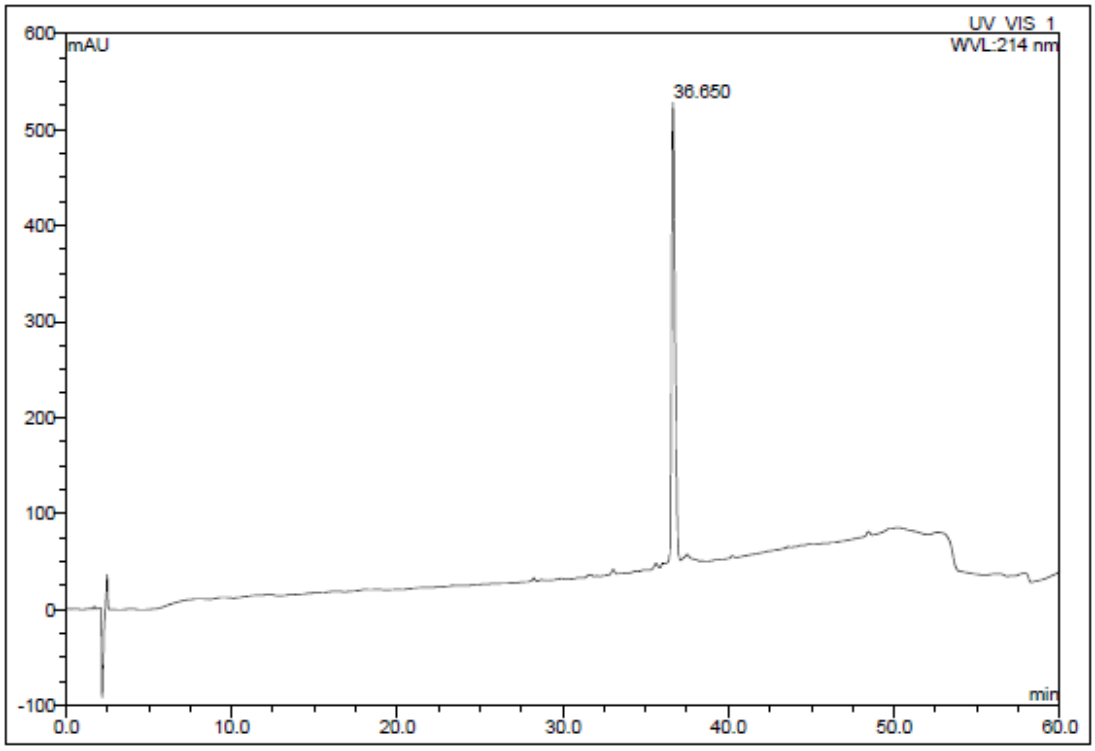
SPPS was carried out as described for mastoparan (**10**). Prior to cleavage 11-azidoundecanoic acid (**189**) was coupled using standard amino acid coupling protocol as described in General Methods. Fmoc deprotection was carried out as described in General Methods.

The peptide was cleaved under standard conditions, washed with ether and lyophilised as described in General Methods. The peptide was purified using a gradient of 5-40% over 30 min at 4 mL min^{-1} using a C18 semi-preparative column. The residue was purified a second time using the same conditions to give (**194**) (6.0 mg, 8%).

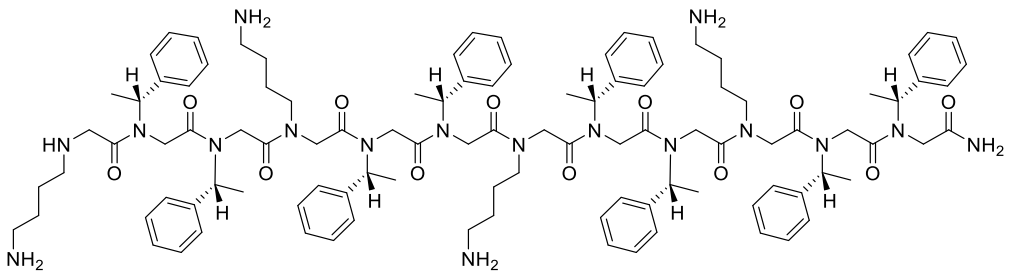
HRMS: (ES$^+$) $[C_{89}H_{152}N_{23}O_{20}]^+$ calculated = 1863.1579; found *m/z* = 932.5844 $[M+2H]^{2+}$, 622.0614 $[M+3]^{3+}$



Analytical HPLC: Standard conditions as described in General Methods. R_f = 36.65 min.



Peptoid (15)

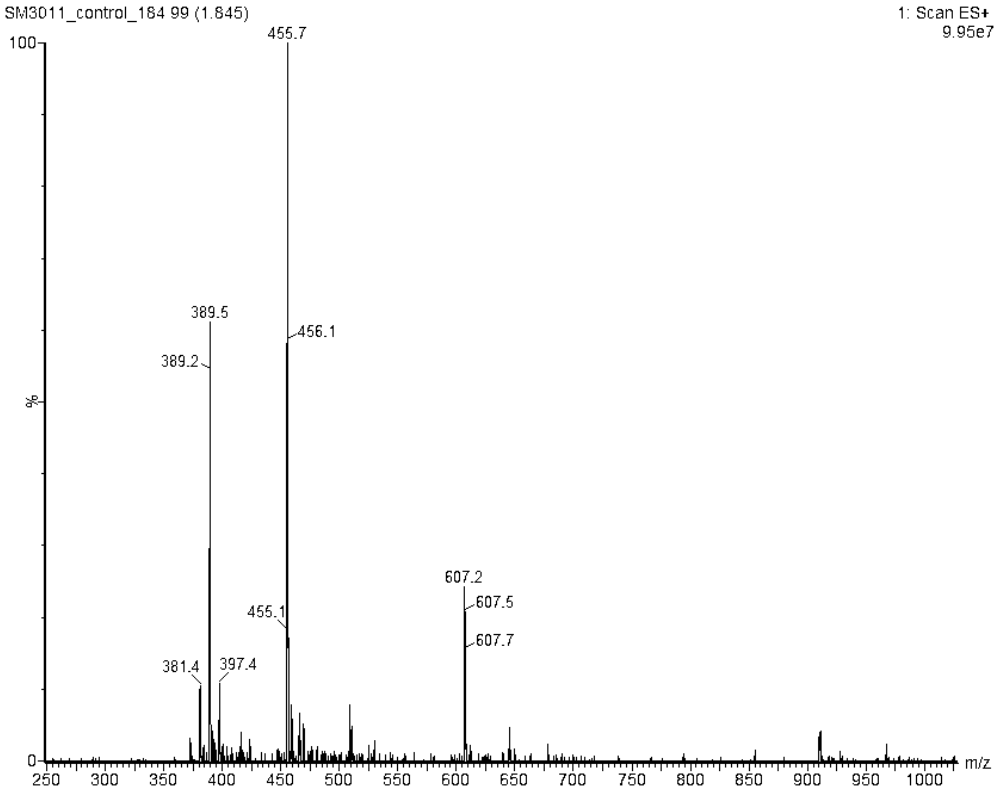


The Rink amide resin (50 mg, 0.7 mmol g^{-1}) was added to a reaction syringe, washed with DMF (4 x 1.5 mL) and left to swell for 20 min in 1.5 mL DMF. After this time, the Fmoc group was removed using the standard Fmoc deprotection step as described in General Methods above. The resin bound amine was then bromoacetylated with the addition of 1.2 M bromoacetic acid in DMF (584 µL) and neat DIC (135 µL) and the coupling was carried out as described in General Methods. Bromide substitution was then carried out with the addition of 1 M MBA (875 µL) and the coupling was carried out as described in General Methods. These two reactions were repeated followed by a third bromoacetylation and the addition of 1 M bocbutanediamine in NMP (891 µL). The coupling was carried out as described in General Methods. The peptoid chain was built up using this submonomer approach with stock solutions of the reagents as listed below.

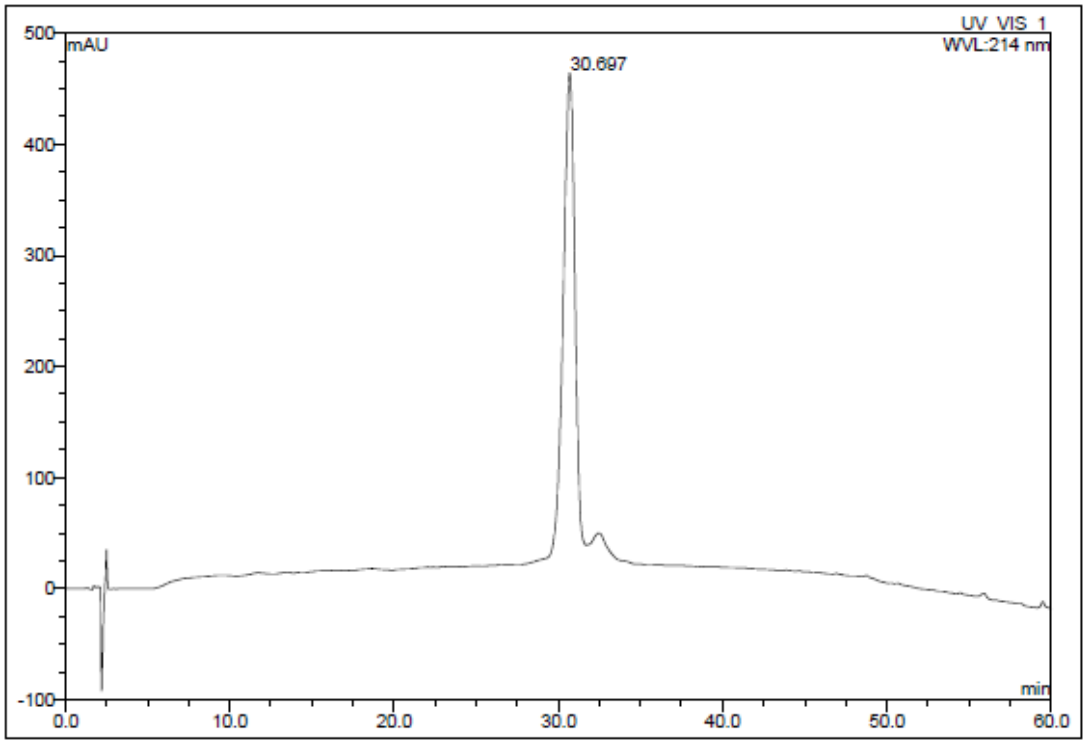
Reagent	Mass (g)	Solvent	Volume (mL)
Bromoacetic acid	1.67	DMF	10.0
DIC	2.04	neat	2.50
Boc-butanediamine	0.941	NMP	5.00
MBA	1.21	NMP	10.0

The peptide was cleaved under standard conditions, washed with ether and lyophilised as described in General Methods. The peptide was purified using a gradient of 5-60% over 30 min at 5 mL min^{-1} using a C18 semi-preparative column. The peptoid was purified a second time using the same conditions to give (**15**) (4.1 mg, 5% yield).

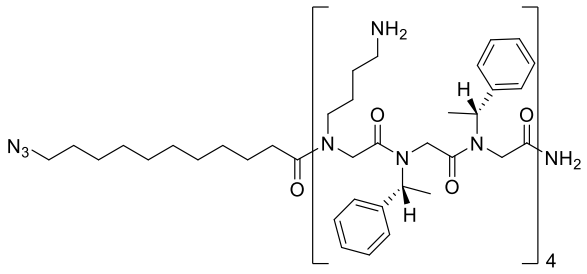
LRMS: (ES$^+$) $[C_{104}H_{140}N_{17}O_{12}]^+$ calculated = 1819.0862; found *m/z* = 607.2 $[M+3H]^{3+}$ 455.5 $[M+4H]^{4+}$



Analytical HPLC: Standard conditions as described in General Methods. R_t = 30.70 min.



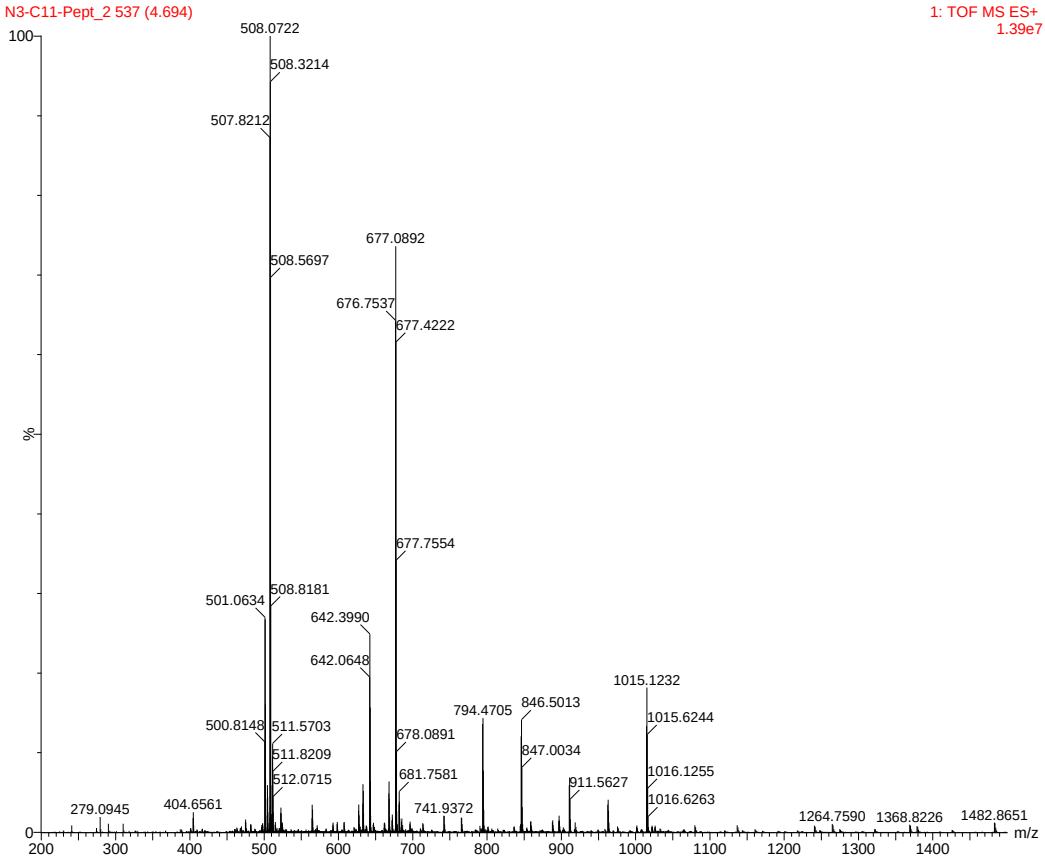
N_3-C11-Peptoid (195)



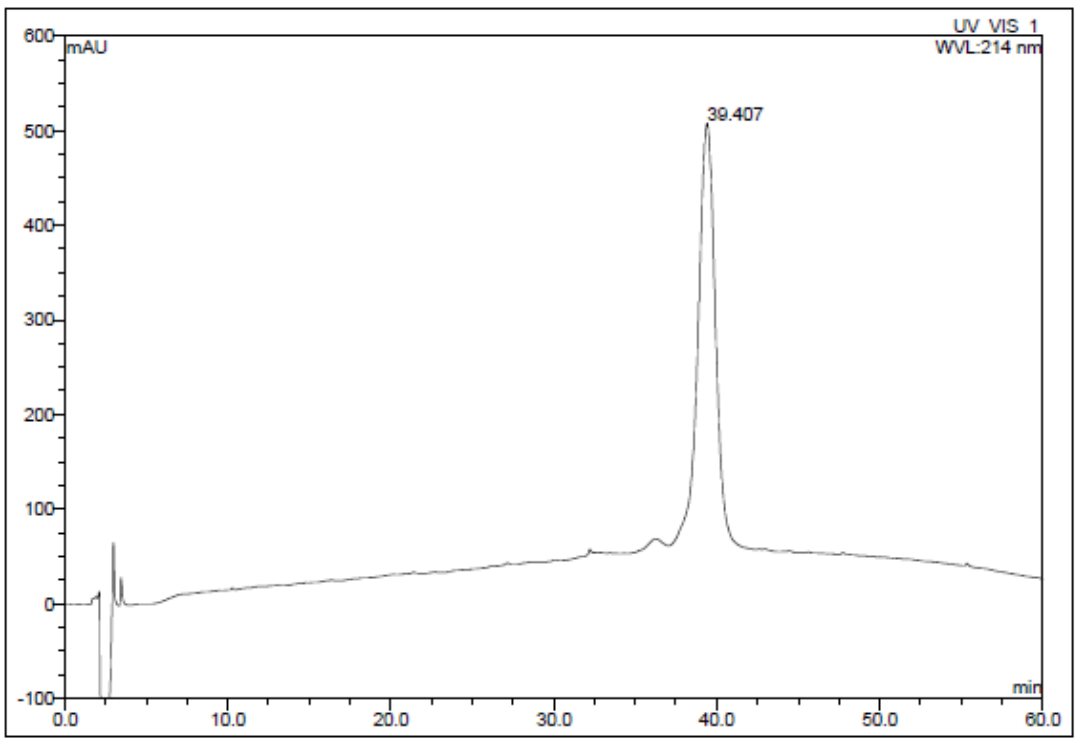
Peptoid synthesis was carried out as described for pexiganan. Prior to cleavage 11-azidoundecanoic acid (**189**) was coupled using standard amino acid coupling protocol as described in General Methods.

The peptide was cleaved under standard conditions, washed with ether and lyophilised as described in General Methods. The peptide was purified using a gradient of 5-60% over 30 min at 5 mL min^{-1} using a C18 semi-preparative column. The peptoid was purified a second time using the same conditions to give (**195**) (6.5 mg, 8%).

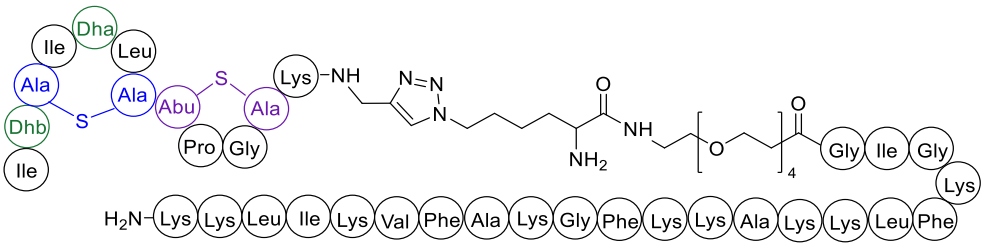
HRMS: (ES$^+$) $[C_{115}H_{159}N_{20}O_{13}]^+$ calculated = 2028.2390; found *m/z* = 1015.1232 $[M+2H]^{2+}$, 677.0892 $[M+3H]^{3+}$, 508.0722 $[M+4H]^{4+}$



Analytical HPLC: Standard conditions as described in General Methods. R_t = 39.41 min.

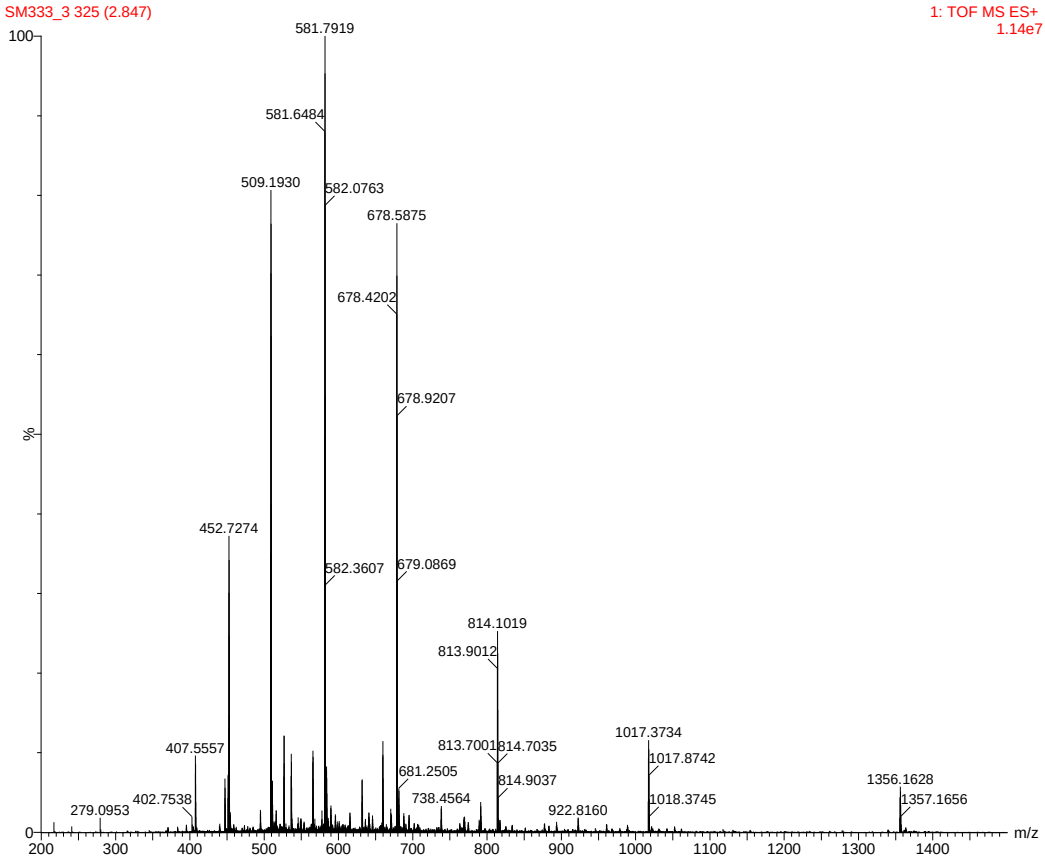


AB-Lys-PEG4-Pexiganan (201)

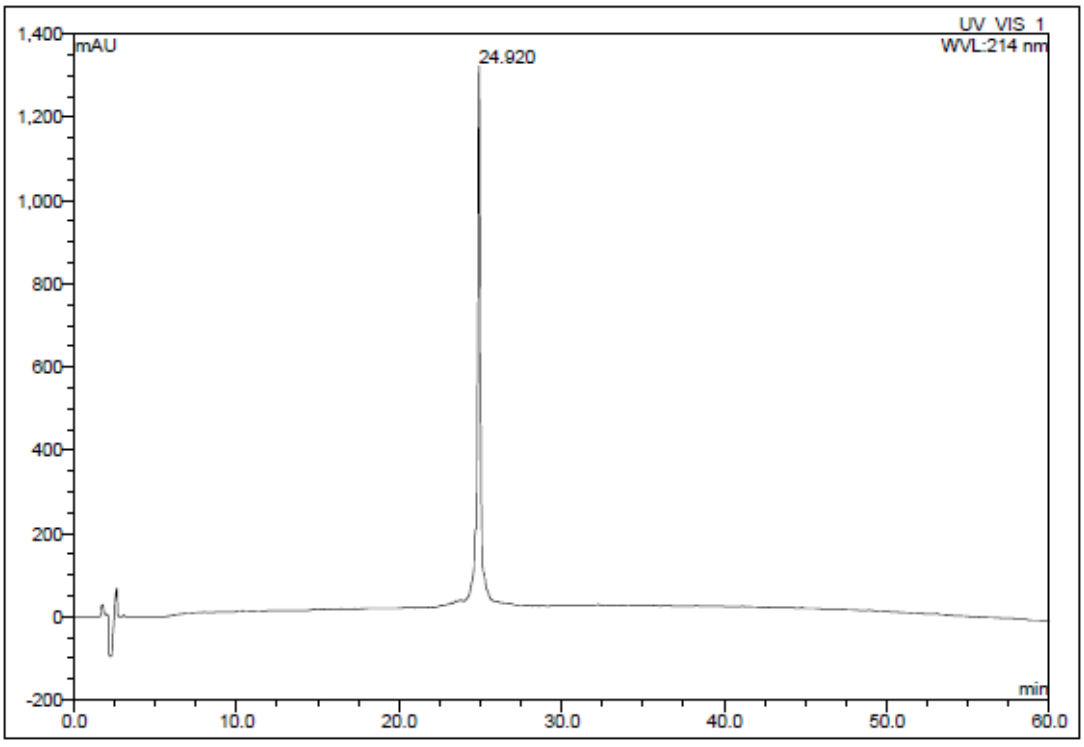


Alkyne (**110**) (1.00 mg, 710 nmol) dissolved in 50 µL of DMF and azide (**183**) (2.8 mg, 710 nmol) dissolved in 50 µL of water were transferred to a 250 µL microwave vial. Stock solutions of $CuSO_4.H_2O$ (42.0 mg, 5.00 mL water) and NaAsc (67.0 mg, 5.00 mL water) and TBTA (2.20 mg, 500 µL DMF) were made up and 50.0 µL was taken from each and added to the reaction vial. The reaction mixture was then irradiated for 20 min at 80 °C. After this time, the reaction mixture was quenched with buffer (3 mL, 0.1% TFA in water), filtered and purified directly using reverse phase HPLC over a gradient of 5-40% over 30 min at 4 mL min^{-1} using a C18 semi-preparative column. The peptide was purified a second time using the same conditions to give clicked product (**201**) (300 µg, 8% yield).

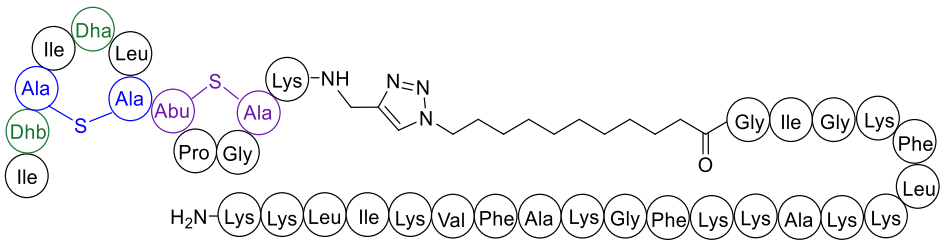
HRMS: (ES$^+$) $[C_{193}H_{328}N_{51}O_{40}S_2]^+$ calculated = 4064.4636; found m/z = 1017.3734 $[M+4H]^{4+}$, 814.1019 $[M+5H]^{5+}$, 678.5875 $[M+6H]^{6+}$, 581.7919 $[M+7H]^{7+}$, 509.1930 $[M+8H]^{8+}$



Analytical HPLC: Standard conditions as described in General Methods. R_t = 29.92 min.

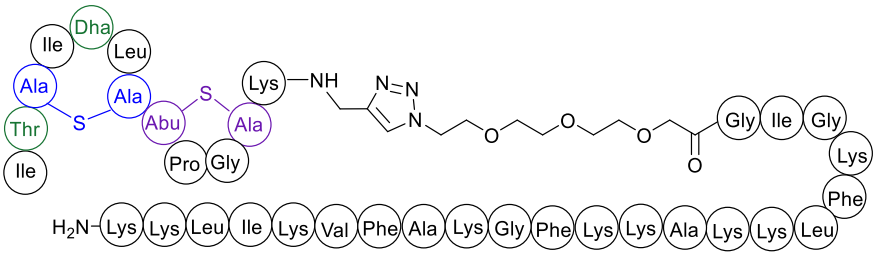


AB-C11-Pexiganan (213)



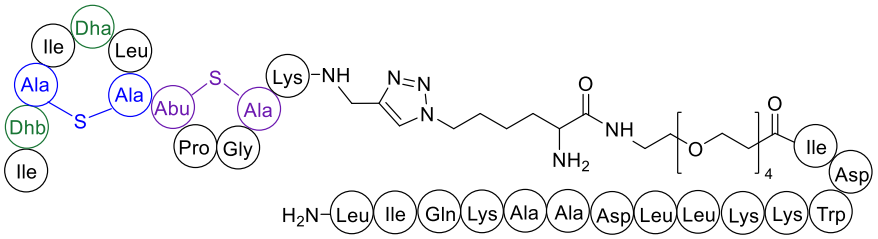
Alkyne (**110**) (1.00 mg, 710 nmol) dissolved in 50 µL of DMF and azide (**193**) (2.56 mg, 710 nmol) dissolved in 50 µL of water were transferred to a 250 µL microwave vial. Stock solutions of $CuSO_4.H_2O$ (42.0 mg, 5.00 mL water) and NaAsc (67.0 mg, 5.00 mL water) and TBTA (2.20 mg, 500 µL DMF) were made up and 50.0 µL was taken from each and added to the reaction vial. The reaction mixture was then irradiated for 20 min at 80 °C. After this time, the reaction mixture was quenched with buffer (3 mL, 0.1% TFA in water), filtered and purified directly using reverse phase HPLC over a gradient of 5-40% over 30 min at 4 mL min^{-1} using a C18 semi-preparative column. No product was observed.

AB-PEG3-Pexiganan (214)



Alkyne (**110**) (1.00 mg, 710 nmol) dissolved in 50 µL of DMF and azide (**191**) (2.30 mg, 710 nmol) dissolved in 50 µL of water were transferred to a 250 µL microwave vial. Stock solutions of $CuSO_4.H_2O$ (42.0 mg, 5.00 mL water) and NaAsc (67.0 mg, 5.00 mL water) were made up and 50.0 µL was taken from each and added to the reaction vial. The reaction mixture was then irradiated for 20 min at 80 °C. After this time, the reaction mixture was quenched with buffer (3 mL, 0.1% TFA in water), filtered and purified directly using reverse phase HPLC over a gradient of 5-40% over 30 min at 4 mL min^{-1} using a C18 semi-preparative column. No product was observed.

AB-Lys-PEG4-Mastoparan (203)



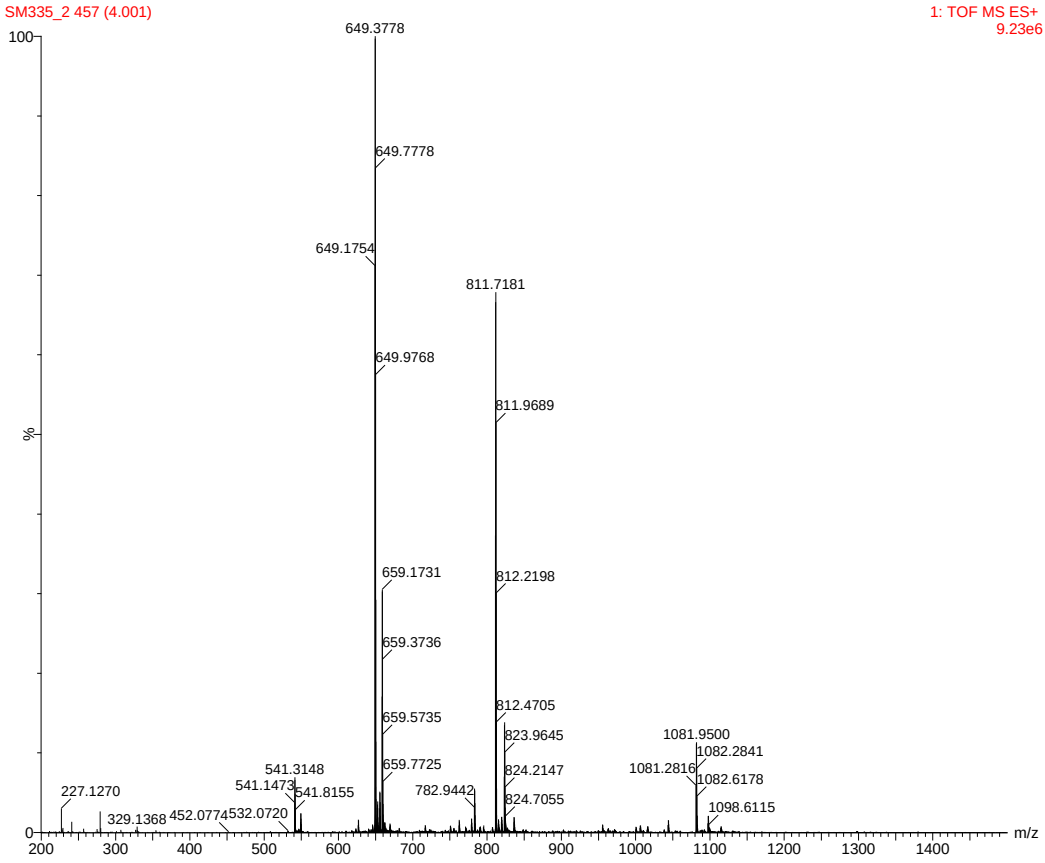
Method 1 - Unsuccessful

Alkyne (**110**) (1.00 mg, 710 nmol) dissolved in 50 µL of DMF and azide (**190**) (2.37 mg, 710 nmol) dissolved in 50 µL of water were transferred to a 250 µL microwave vial. Stock solutions of $CuSO_4.H_2O$ (42.0 mg, 5.00 mL water) and NaAsc (67.0 mg, 5.00 mL water) were made up and 50.0 µL was taken from each and added to the reaction vial. The reaction mixture was then irradiated for 20 min at 80 °C. After this time, the reaction mixture was quenched with buffer (3 mL, 0.1% TFA in water), filtered and purified directly using reverse phase HPLC over a gradient of 5-40% over 30 min at 4 mL min^{-1} using a C18 semi-preparative column. No product was observed.

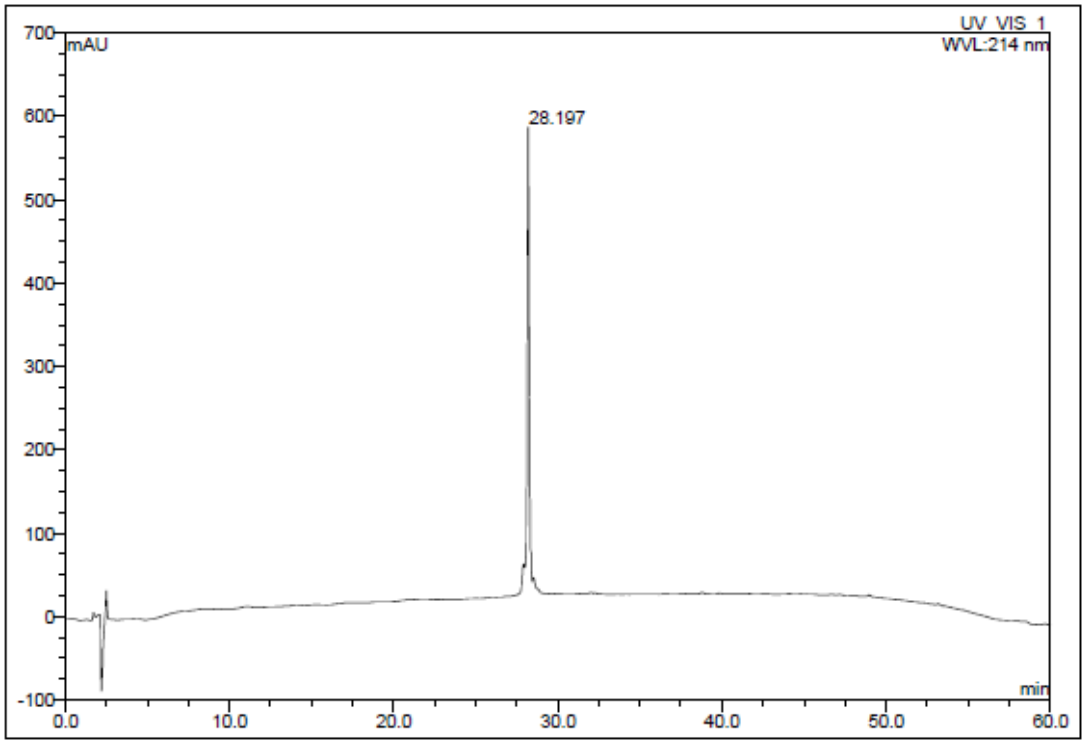
Method 2 - Successful

Alkyne (**110**) (1.00 mg, 710 nmol) dissolved in 50 µL of DMF and azide (**190**) (2.37 mg, 710 nmol) dissolved in 50 µL of water were transferred to a 250 µL microwave vial. Stock solutions of $CuSO_4.H_2O$ (42.0 mg, 5.00 mL water) and NaAsc (67.0 mg, 5.00 mL water) and TBTA (2.20 mg, 500 µL DMF) were made up and 50.0 µL was taken from each and added to the reaction vial. The reaction mixture was then irradiated for 20 min at 80 °C. After this time, the reaction mixture was quenched with buffer (3 mL, 0.1% TFA in water), filtered and purified directly using reverse phase HPLC over a gradient of 5-40% over 30 min at 4 mL min^{-1} using a C18 semi-preparative column. The peptide was purified a second time using the same conditions to give clicked product (**203**) (300 µg, 9% yield).

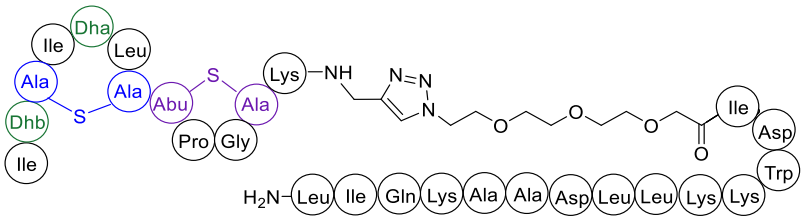
HRMS: (ES$^+$) $[C_{149}H_{250}N_{39}O_{37}S_2]^+$ calculated = 3241.8316; found *m/z* = 1081.9500 $[M+3H]^{3+}$, 811.7181 $[M+4H]^{4+}$, 649.3778 $[M+5H]^{5+}$



Analytical HPLC: Standard conditions as described in General Methods. R_t = 28.20 min.

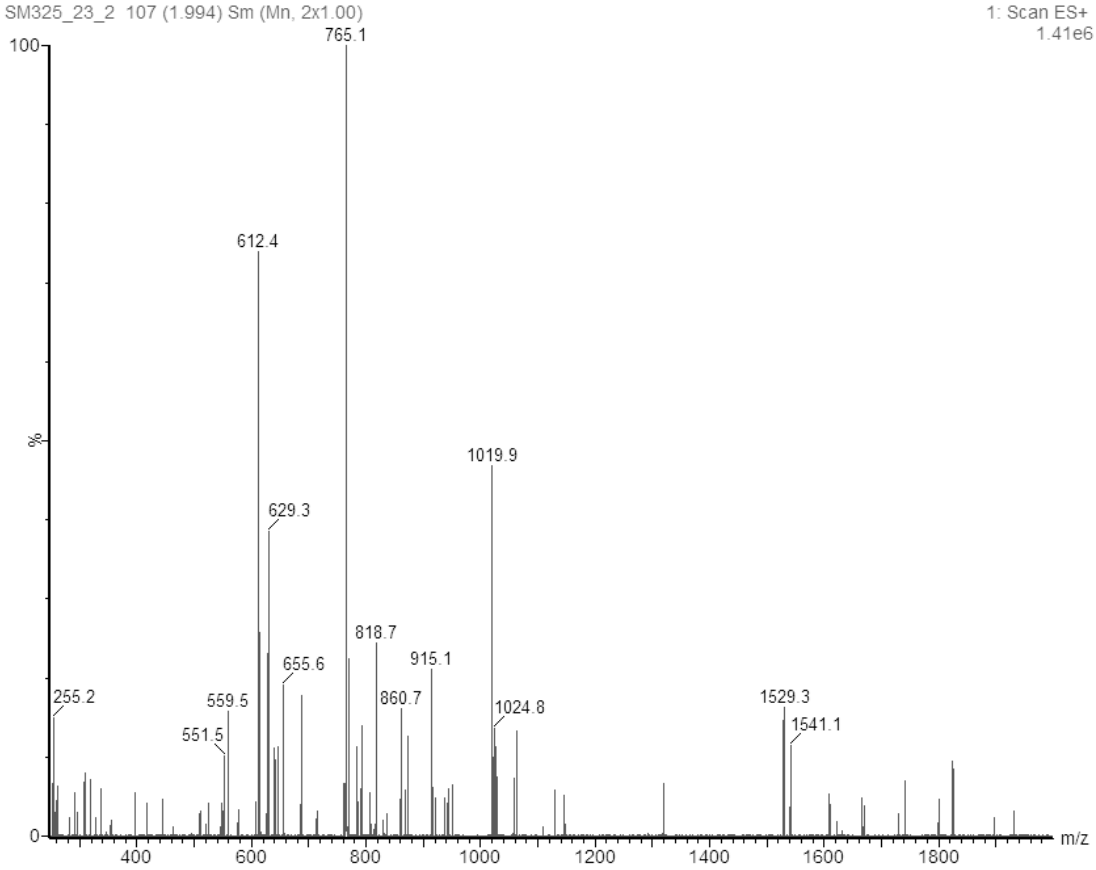


AB-PEG3-Mastoparan (204)

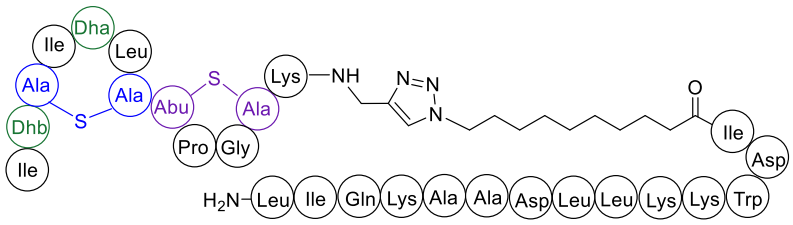


Alkyne (**110**) (1.00 mg, 710 nmol) dissolved in 50 µL of DMF and azide (**192**) (1.57 mg, 710 nmol) dissolved in 50 µL of water were transferred to a 250 µL microwave vial. Stock solutions of $CuSO_4.H_2O$ (42.0 mg, 5.00 mL water) and NaAsc (67.0 mg, 5.00 mL water) and TBTA (2.20 mg, 500 µL DMF) were made up and 50.0 µL was taken from each and added to the reaction vial. The reaction mixture was then irradiated for 20 min at 80 °C. After this time, the reaction mixture was quenched with buffer (3 mL, 0.1% TFA in water), filtered and purified directly using reverse phase HPLC over a gradient of 5-40% over 30 min at 4 mL min^{-1} using a C18 semi-preparative column. The peptide was purified a second time using the same conditions to give clicked product (**204**) (120 µg, 5% yield).

LRMS: (ES$^+$) $[C_{140}H_{232}N_{37}O_{35}S_2]^+$ calculated = 3055.6948; found *m/z* = 1019.9 $[M+3]^{3+}$, 765.1 $[M+4H]^{4+}$, 612.4 $[M+5H]^{5+}$



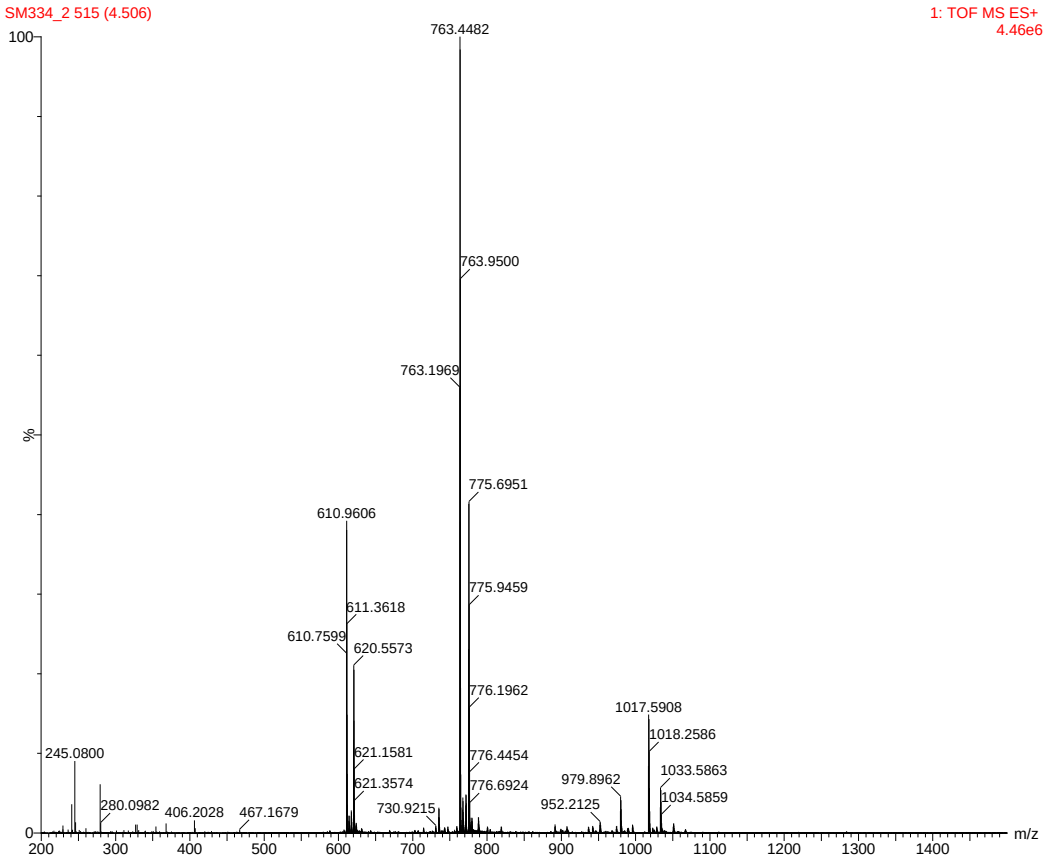
AB-C11-Mastoparan (205)



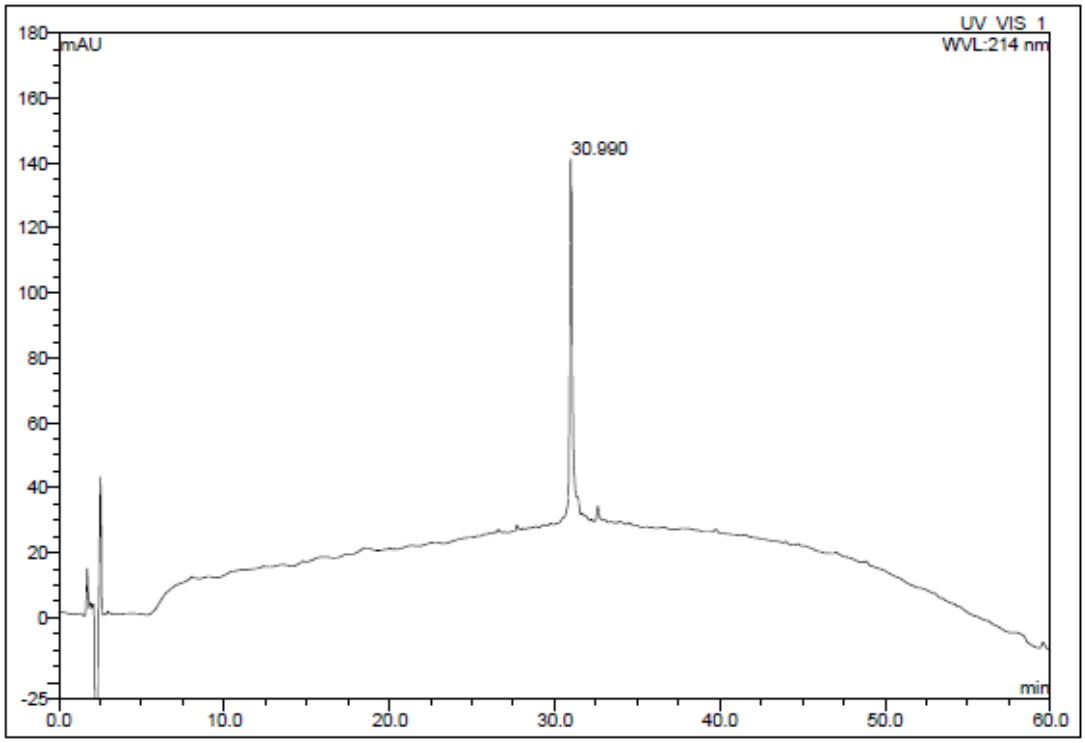
Alkyne (**110**) (1.00 mg, 710 nmol) dissolved in 50 µL of DMF and azide (**194**) (1.57 mg, 710 nmol) dissolved in 50 µL of water were transferred to a 250 µL microwave vial. Stock solutions of $CuSO_4.H_2O$ (42.0 mg, 5.00 mL water) and NaAsc (67.0 mg, 5.00 mL water) and TBTA (2.20 mg, 500 µL DMF) were made up and 50.0 µL was taken from each and added to the reaction vial. The reaction mixture was then irradiated for 20 min at 80 °C. After this

time, the reaction mixture was quenched with buffer (3 mL, 0.1% TFA in water), filtered and purified directly using reverse phase HPLC over a gradient of 5-50% over 40 min at 5 mL min^{-1} using a C18 semi-preparative column. The peptide was purified a second time to give clicked product (**205**) (200 µg, 8% yield).

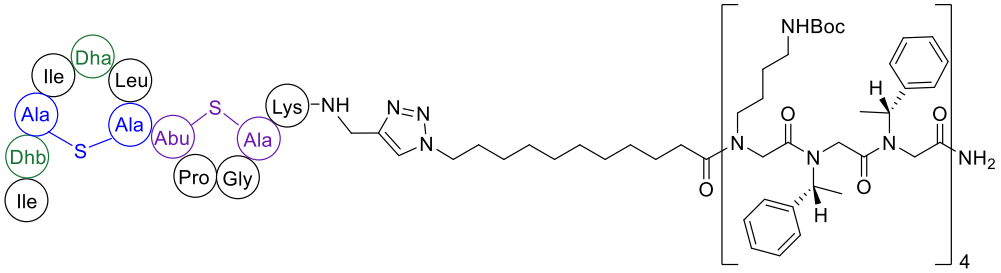
HRMS: (ES^+) $[C_{143}H_{238}N_{37}O_{32}S_2]^+$ calculated = 3049.7570; found *m/z* = 1017.5908 $[M+3H]^{3+}$, 763.4482 $[M+4H]^{4+}$, 610.9606 $[M+5H]^{5+}$



Analytical HPLC: Standard conditions as described in General Methods. R_t = 30.99 min.

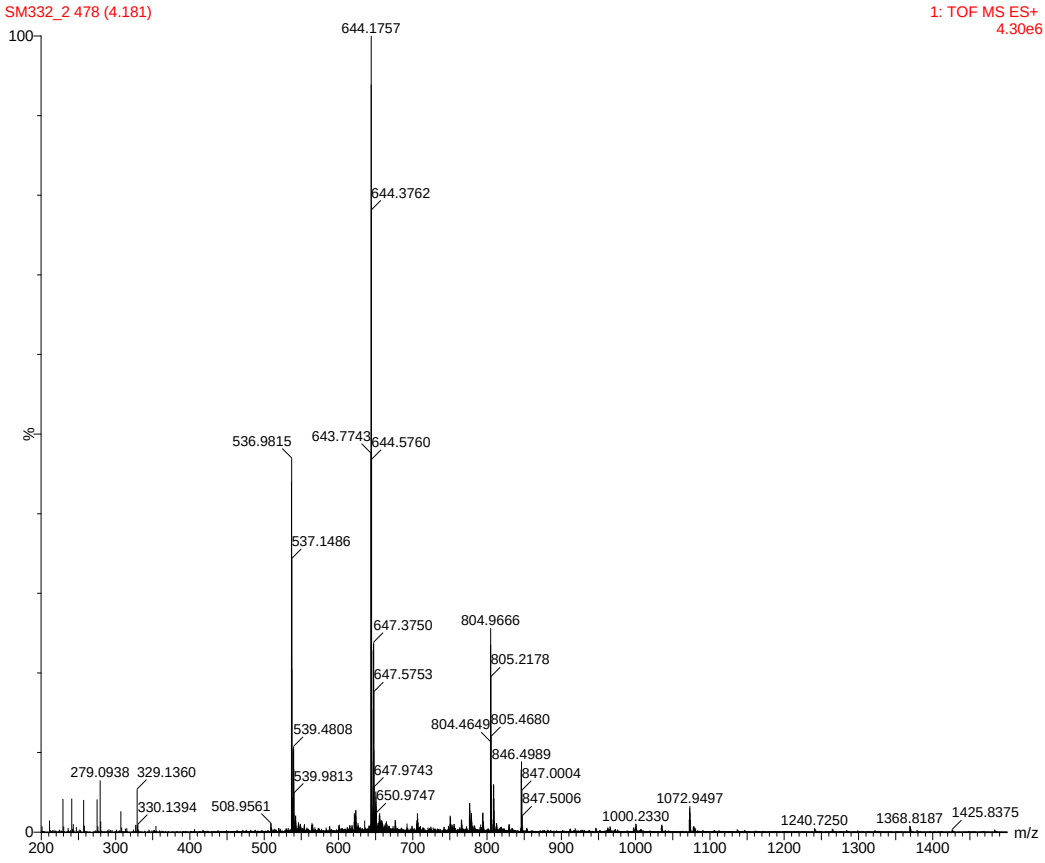


AB-C11-Peptoid (206)

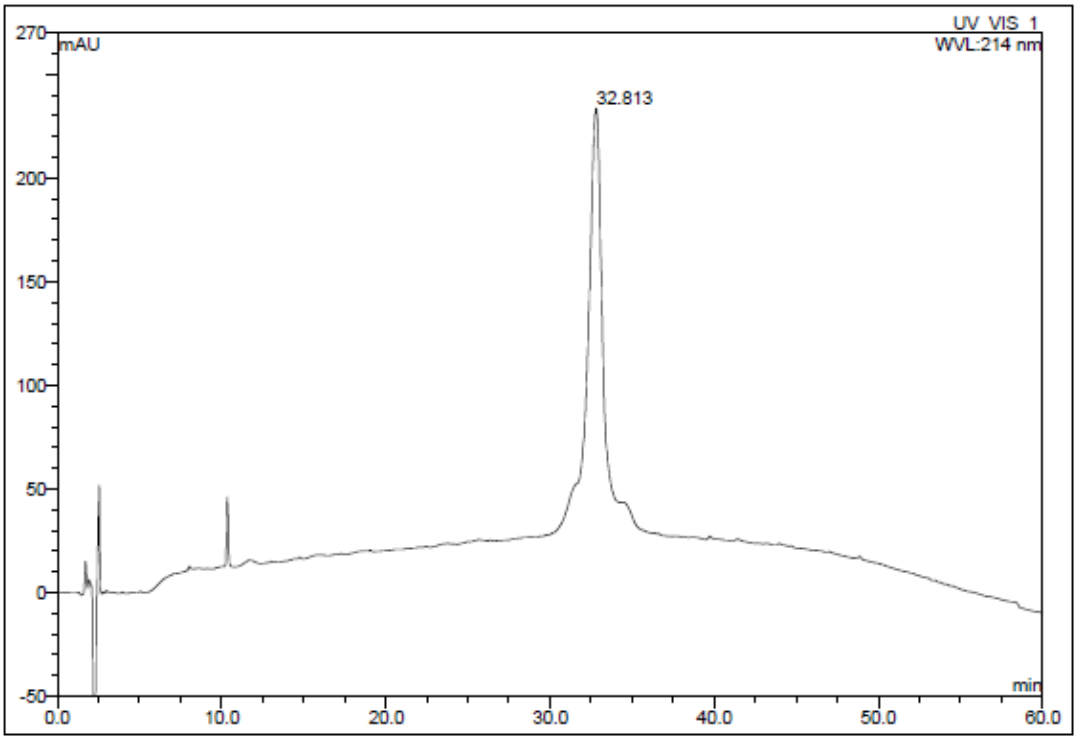


Alkyne (**110**) (1.00 mg, 710 nmol) dissolved in 50 µL of DMF and azide (**195**) (1.76 mg, 710 nmol) dissolved in 50 µL of water were transferred to a 250 µL microwave vial. Stock solutions of $CuSO_4.H_2O$ (42.0 mg, 5.00 mL water) and NaAsc (67.0 mg, 5.00 mL water) and TBTA (2.20 mg, 500 µL DMF) were made up and 50.0 µL was taken from each and added to the reaction vial. The reaction mixture was then irradiated for 20 min at 80 °C. After this time, the reaction mixture was quenched with buffer (3 mL, 0.1% TFA in water), filtered and purified directly using reverse phase HPLC over a gradient of 5-60% over 30 min at 5 mL min^{-1} using a C18 semi-preparative column. The peptide was purified a second time using the same conditions to give clicked product (**206**) (250 µg, 9% yield).

HRMS: (ES$^+$) $[C_{169}H_{245}N_{34}O_{25}S_2]^+$ calculated = 3214.8381; found *m/z* = 1072.9497 $[M+3H]^{3+}$, 804.9666 $[M+4H]^{4+}$, 644.1757 $[M+5H]^{5+}$, 536.9815 $[M+6H]^{6+}$



Analytical HPLC: Standard conditions as described in General Methods. R_t = 32.81 min.



7.6 Experimental for *in vitro* Biology

7.6.1 General Methods

In vitro work was carried out under sterile conditions in a category 2 safety hood. Oxoid Nutrient Broth No 2 was used purchased from ThermoFisher Scientific as a dehydrated powder and was made up as a solution according to the manufacturer's instructions. OD600 readings of bacteria stock solutions were taken using a Nanodrop 2000c Spectophotometer. Polypropylene and polystyrene 96-well plates were purchased from Greiner Bio One. Aeraseal Sterile gas permeable seals were purchased from Excel Scientific. 96-Well plates were incubated overnight in Eppendorf Thermomixer comfort/C incubators. OD625 readings of 96-well plates were measured using a Tecan infinite M200 Pro plate reader with i-control 1.11 software.

7.6.2 Well Diffusion Method

All compounds to be tested were made up to a concentration of 1 mg mL^{-1} in sterile water except sodium trifluoroacetate which was made up to 0.4 mg mL^{-1}. LB agar plates were

inoculated with 100 µL of an overnight culture of either *Micrococcus luteus*, *Bacillus subtilis*, *Escherichia coli* or *Pseudomonas aeruginosa* and spread evenly across the surface of the plate. The overnight cultures of bacteria were grown at 37 °C in Oxoid Nutrient Broth No. 2 with shaking. Four wells of 8 mm diameter were punched into each plate. 25 µL of the stock solution was spotted into a well for each strain of bacteria. Plates were incubated for 48 h at 37 °C except for those inoculated with *M. luteus* which were incubated at 30 °C. Later work with *M. luteus* was carried out at 37 °C. After 48 h, antibacterial activity was evaluated by the appearance of a zone of inhibition.

7.6.3 Broth Micro-dilution Method

Overnight stocks of bacteria were prepared by Prof John Ward. Bacteria were streaked out onto nutrient agar plates and single colonies taken from these to plates to grow overnight in 5 ml Nutrient broth No.2 (Oxoid) with shaking at 37 °C. The absorbance of the bacterial cultures were measured at 600 nm and diluted by a factor of 10 with nutrient broth to give an OD of 0.118. Routinely 10 µL of this dilution was used in the micro titre plates to test the antimicrobial activity of the compounds.

Oxoid Nutrient Broth No 2 was used with 1% DMSO to aid solubilisation. All compounds to be tested were made up to 0.60 mM stock solutions in sterile water with 1% DMSO. To ensure peptides were fully dissolved stock solutions were placed in a bath sonicator for 30 s. 10 µL of peptide stock solution was added to 90 µL of nutrient broth with 1% DMSO. This was then transferred to the first column of a 96-well polypropylene plate. A two-fold serial dilution was carried out as described by Hancock and co-workers:[233] 50 µL of nutrient broth was added to columns 2-10. 50 µL was taken from column 1 and added to column 2 and mixed well by pipetting up and down 8 times. 50 µL was taken from column 2 and added to column 3 and again mixed well. This process was repeated through to column 10. The 50 µL taken from 10 was discarded. 250 µL of nutrient broth was added to the sterility control column, 12, and 240 µL was added to the growth control column, 11.

10 µL of an overnight stock of bacteria adjusted to have an OD 0.817 (of a 1 in 10 dilution) was added to each well except from the final column which was reserved as a sterility control containing only nutrient broth. A further portion of Oxoid Nutrient Broth No 2 was added to make each well up to 250 µL total volume. A gas permeable adhesive seal was applied to each plate and they were incubated for 20 h at 37 °C. After this time broth and

bacteria were re-suspended and transferred to clear, flat-bottomed, polystyrene 96-well plates and the OD was measured. The MIC is defined as the lowest concentration of the antimicrobial agent that inhibits visible growth of the tested isolate as observed with the unaided eye. The error of the MIC results is estimated as one half of the interval between the MIC dilution and the next lowest concentration (i.e. the highest concentration that resulted in growth of bacteria).

Colony counts were carried out by Prof John Ward. The viable cell numbers were calculated by using a dilution series of the overnight culture to a final of 10^{-7} and plating 100 µL aliquots of the 10^{-6} and 10^{-7} dilutions on nutrient agar plates (2 per dilution) and incubating overnight. The viable cell count in the original overnight culture was calculated from the numbers of colonies on these plates x the amount plated x the dilution factor and expressed as viable cells per mL.

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